Characterization of the *Bambusa tulda* Roxb. inflorescences and flowers at the morphological, anatomical and genetic levels to understand floral organ developments and differentiation in bamboo

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Abstract

Bamboos belong to the monocotyledonous plant family Poaceae and subfamily Bambusoideae. Bamboos represent an extreme example of perennialism, where the reproductive phase begins after completion of an extended vegetative phase which may vary across species. Other than delayed flowering time, existence of two different types of inflorescences, genetics of floral development and pollen compatibility make bamboo unique in comparison to other grasses.

Therefore, the main aim of this study is to understand the floral organ development and pollination mechanism in *Bambusa tulda*. . In this study, the relative abundance of two different types of inflorescence in an entire flowering cycle were analysed and rate of seed setting was compared. One potential limitation to perform gene expression analyses on *Bambusa* members was the absence of suitable reference genes for data normalization. Therefore, reference genes were identified, which were stable across species, organs and developmental stages. In order to understand the molecular mechanism regulating flower development, an important group of floral meristem identity genes known as MADS box gene family were analyzed in five bamboo genomes using *in silico* tools. The *APETALA1/FRUITULL* gene *MADS14* and MADS15, were sequenced from *Bambusa tulda* and their expression pattern was analyzed across different vegetative and reproductive tissues. In order to understand the genetics of pollen compatibility in bamboo, *in vivo* pollination experiments were performed, which indicated that *B. tulda* is primarily a cross pollinated species. In order to further understand the molecular mechanism regulating genetic compatibility, members of RNase T2 and *Fbox* gene families were studied. Overall, the findings of this study will be useful to understand the reproductive nature and flower development in bamboos.

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1. Introduction

Bamboos are the giant grass species that grows both in tropical and temperate regions of the world (Trujillo, 2007). Monocarpy dominates in majority bamboo species along with a long vegetative phase, which may be extended up to 120 years (Janzen, 1976). Bamboo flowering is recognized as a bad omen primarily due to increase in rodent population immediately after flowering, which results into massive crop loss (Mohan Ram and Hari Gopal, 1981; John and Nadgauda, 2002).

In grasses, spikelet inflorescence containing florets is the primary structural unit having sterile bracts in the outer whorls and male and female organs in the inner whorls (Yoshida and Nagato, 2011). Unlike grasses, bamboos bear two different types of inflorescences- true spikelet and pseudospikelet (McClure 1966). The true spikelet represents a single spikelet unit whereas, pseudospikelet is composed of cluster of multiple spikelets arising from a node (Chakraborty et al., accepted for publication). Even though, there is no apparent difference with respect to overall development and morphology of these two inflorescence types, their evolutionary and reproductive significance has not been understood.

Flower induction happens as a result of conversion of shoot apical meristem (SAM) to inflorescence meristem (IM) and ultimately spikelet meristem. At the molecular level, this conversion of SAM to IM is primarily mediated by MADS box family proteins (Kobayashi et al., 2012). Members of the MADS box gene family are a large group of transcription factors, characterized by a highly conserved MADS domain. Other than the MADS domain, additional plant specific domains are present which are a less conserved I (intervening) domain, a conserved K (keratin like coiled coil) domain and a variable C terminal domain (Ma et al., 1991). The highly conserved MADS domain primarily acts as a DNA binding domain in addition to its role in protein dimerization and nuclear localization of MADS box proteins (Gramzow and Theißen, 2010). Members of MADS box family are homeotic genes, which are involved in organ development explained by the ABCDE model (Theissen, 2001). Among the ABCDE genes, the A family is composed of *APETALA1/FRUITFULL* (*AP1/FUL*) like genes and are primarily involved in the early stages of meristem specification and inflorescence development. For instance, rice genome contains four *AP1/FUL* like genes, which are *MADS14*, *15, 18* and *20*

(Callens et al., 2018). In spite of their enormous biological significance, not much studies have been carried out to decipher their roles in the IM specification and flower development in bamboos (Tian et al. 2005, Lin et al. 2009, Shih et al. 2014, Zhu et al. 2016, Cheng et al., 2017, Zhang et al. 2018).

Reproductive success of a plant no only depend on the proper development of inflorescence and floral organs, but also on successful fertilization and seed set. Despite having different inflorescence types, the sexual reproduction is less frequent in bamboos and they are primarily dependent on vegetative propagation. In order to increase genetic variability, plants have adopted self incompatibility (SI), where only non-self pollens germinate on the stigma (Kao and Tsukamoto, 2004). This compatibility between stigma and pollen during fertilization is an important determining factor for successful fertilization. Bamboos display variations in their compatibility whether self or cross compatible, primarily based on availability of pollen grains and environmental factors (Kitamura and Kawahara, 2011, Mizuki et al., 2014, Xie et al., 2019). Grasses display gametophytic self incompatibility (GSI) where, the SI nature of pollen is governed by genotype of the haploid microspores and SI reaction occurs when the *S* haplotypes of the pollen matches with both the *S* haplotypes present in diploid pistil (Silva and Goring, 2007). Unlike dicotyledonous plants where GSI is regulated by a single *S* locus, in grasses, GSI is maintained by two un-linked, multi allelic loci *S* and *Z* (Langridge and Baumann, 2008). Since both *S* and *Z* loci are multi allelic, this allelic diversity can be an important determining factor during a compatibility reaction. Thus in order to maintain genetic diversity, bamboos need to fine-tune their preference for compatible partner so that this can ultimately lead to successful fertilization and seed set. Although the *S*-Z based GSI is known in grasses, information regarding the genes and their molecular regulation are scanty and in Bambusoideae they are even rarer.

One of the most characterized GSI is mediated by SRNase based mechanism where SI reaction is controlled by two tightly linked genes present in a polymorphic gene locus called *S* locus. The pistil specific gene is *SRNase*, which is a member of the RNase T2 family (Golz et al., 1998; Vieira et al., 2008). On the other hand, *S locus F box* (*SLF*) gene, a member of the F box protein family, is the pollen determinant in the GSI system. In addition to the conserved domains in SRNase and SLF, both of these proteins have hypervariable regions that determine their allele specificity during SI interaction (Vieira and Charlesworth, 2002; Ikeda et al., 2004).

Nevertheless, no information is available regarding the molecular regulation of GSI in Bambusoideae sub family. Thus, it is important to identify and characterize homologous bamboo genes and study their expression in different tissues and developmental time points to understand if such mechanisms are also active in bamboo or not.

2. Objectives (as specified in approved 1000 words)

Objective I. Identification of stable reference genes in bamboo suitable for gene expression analyses

- Selection of candidate reference genes for gene expression analyses
- Analysis of RT-qPCR efficiency by standard curve method using serial dilution of the cDNA solutions
- Study of the expression stability of the candidate reference genes across four different species of *Bambusa* by real time qRT-PCR analyses
- Study of the expression stability of candidate reference genes in nine different organs of *B. tulda* by real time qRT-PCR analyses
- Study of the expression stability of candidate reference genes in different developmental stages of three selected organs of *B. tulda*
- *In silico* study to analyze the stability of the studied reference genes using geNorm, NormFinder and RefFinder tools
- Validation of the stability of selected reference genes using two flower associated target genes

Objective II. A comparison between spikelet vs pseudospikelet and investigations to understand the development of anthers, ovary and setting of seeds in the inflorescences of *B. tulda* Roxb.

- Study on the frequency of spikelet vs. pseudospikelet in an entire flowering cycle of *B. tulda*

- Study on the developmental progression of vegetative and inflorescence buds of *B. tulda* by scanning electron microscopy
- Microscopic observations on the stages of anther developments in the spikelet of *B. tulda*
- Microscopic observations on the stages of ovary developments in the spikelet of *B. tulda*
- A comparative analysis on the frequency of seed production in the spikelet and pseudospikelet of *B. tulda*

Objective III. Study on the pollen-pistil interactions and identification of genes regulating this process in bamboo

- *In vivo* self and cross pollination analyses to study self- compatibility vs. incompatibility in *B. tulda*
- PCR isolation, sequencing and molecular characterization of *S-RNase* and *F-box* genes from *B. tulda*
- Study on the phylogenetic relationship of *S-RNase* and *F-box* genes in comparison to the homologous sequences from related monocot species
- Tissue specific expression analyses of S-*RNase* and *F-box* genes by real time RTq-PCR analyses

Objective IV. *In silico* identification and sequence characterization of floral organ specific MADS box family genes in bamboo and other grasses

- PCR isolation, sequencing and molecular characterization of *BtMADS14, 15* and *18* genes
- Study on the phylogenetic relationship of *BtMADS14, 15* and *18* genes in comparison to the homologous sequences from related monocot species
- Tissue specific expression analyses of *BtMADS14, 15* and *18* genes by real time RTq-PCR analyses

- *In silico* analyses of promoter sequences in comparison to homologs from other closely related monocot species
- *In silico* analyses of structural motifs in comparison to homologs from other closely related monocot species

3. Methodology

3.1. Collection of tissues for microscopy analyses

In order to perform various microscopic analyses on inflorescence buds and floral organs, fresh tissues were collected from three populations of *Bambusa tulda* Roxb. growing naturally in Shyamnagar (SHYM7, 22.830829°N, 88.405029°E; SHYM16, 22.829591°N, 88.409095°E) and Bandel (BNDL23, 22.934348°N, 88.353255°E), West Bengal, India.. Both vegetative bud and inflorescence buds from two developmental stages of spikelet, early and middle along with young bud from pseudospikelet were selected for scanning electron microscopy study. In addition, late staged inflorescence buds were also collected to study the developmental progression of anther and ovary.

3.2. Collection of mature inflorescences to compare seed setting rate between spikelet and pseudo-spikelet inflorescence

In order to study difference in the rate of seed production between true spikelet and pseudospikelet, both types of mature inflorescences were collected from ~7-12 culms from each of the three different populations as mentioned in section 3.1 during February to May, 2018. Seeds were counted from 96 true spikelets and 99 spikelet units obtained from 120 pseudospikelet. Both true and pseudospikelets were randomly selected from the flowering branches of ~6-7 culms from a clump. In order to make the analysis comparable, only spikelet containing 7-9 florets were selected from each of the inflorescence types. In case of true spikelet, total number of seeds produced by all the florets within a spikelet were counted. On the other hand, for pseudospikelet, only the fully developed spikelets with similar floret number located within that cluster were selected to count the total number of seeds.

3.3. Scanning electron microscopic (SEM) analysis

In order to perform SEM analyses, the outer protective layers of vegetative and inflorescence buds were carefully removed to expose the meristem tip, coated them in platinum using POLARON-SC7620, Carbon Accessory (Model-CA76) and were observed in ZEISS EVO 18 Scanning Electron Microscope (Carl Zeiss SMT, Germany) with maximum acceleration voltage of 30kV. The microscopy facilities of Central Research Facility (CRF), IIT-Kharagpur; University Science Instrument Centre, University of Burdwan and Centre for Research in Nanoscience and Nanotechnology (CRNN), University of Calcutta were availed to perform SEM analyses.

3.4. *In vivo* self and cross pollination experiment

In order to understand the nature of pollen-pistil interaction in *B. tulda*, self and cross pollination experiments were performed in their natural habitat. Two geographically distant (~350 m) flowering populations (BNDL23 and BNDL24, 22.932155°N, 88.355551°E) located in Bandel, West Bengal, India, were selected for this study. For an individual population, ~7-8 culms were used for this study. Approximately, 30 spikelets obtained from each population were used for pollination experiments. Prior to pollination, the selected flowers were emasculated and pollens were dusted within 15mins to ensure that their viability remained intact. The time to observe maximum number of open florets was observed for three successive days in both populations at five different time points- 6 am, 8 am, 10 pm, 12 pm and 2 pm. Further, to identify the optimum time of pollen germination, pollination experiments were performed at four different time points- 8 am, 10 am, 12 pm and 2 pm. Pollinated stigmas were collected at 2 and 4 hours post pollination and after successive treatments and staining, they were observed under bright field microscope (Carl Zeiss, Axiostar Plus, Germany). Pollen grains germinated on stigma surface were counted. For both self and cross pollination experiments, pollination was performed at 10am and the pollinated pistils were collected at 12pm. In case of self pollination, pollens obtained from matured anthers of one population were dusted on the stigma of an open flower located in the same clump. On the other hand, cross pollination was performed by dusting pollens obtained from one population to the stigma in other population. The pollinated flowers were immediately covered with plastic bags to prevent further pollination. The rate of germination frequency was calculated by diving the total number of germinated pollens to the total number of pollens.

3.5. Collection of tissues for isolation of nucleic acid

Different flowering and vegetative tissues of *B. tulda* were collected from three different geographic locations as mentioned in section 3.1. Young and green leaves were used for genomic DNA isolation. In order to identify suitable reference gene for gene expression analysis, three widely grown tropical bamboo species *B. balcooa* Roxb., *B. bambos* L., and *B. vulgaris* Schrad. ex Wendl. were selected. To study the influence of organ variability on the stability of reference genes, young leaves from flowering (YLF) and non-flowering (YLN) culms, flag leaves (FL, leaf just below the mature inflorescence), possible flag leaves (PFL, leaf covering the immature inflorescence), culm sheaths (CS), internodes (I), roots (R), rhizomes (RH), and early staged inflorescence buds (E) were obtained from *B. tulda*. In order to study the impact of physiological age, tissues were obtained from three different internode positions (apical, middle and basal), and leaves (apical, middle and basal). In addition, inflorescence buds having different developmental stages (early, middle and late) were collected. To study the impact of species variation on the expression stability of candidate reference genes, three representative vegetative organs-rhizome, young leaves and internodes were collected from all the four bamboo species. The gene amplification and expression analyses of three *AP1* like genes were performed in eight different vegetative and three floral bud tissues of *B. tulda*. However, expression analysis on anther and pistil specific genes putatively involved in pollen pistil interaction were conducted by using four vegetative (leaf; L, internode, rhizome, culm sheath) and three reproductive (floral bud; F, anther; A and pistil; P) tissues. Three biological replicates were considered for each tissue stages studied. Freshly harvested tissues were quickly snap frozen in liquid nitrogen and stored in -80ºC freezer. All the voucher specimens were submitted to the Botanical Survey of India (B.S.I), Shibpur herbarium (voucher nos. 56A, 56B, 57A, 57B, 58A. 58B, 59A, 59B, 59C on 05.06.2015).

3.6. Retrieving genomic and protein sequences from databases

In order to design primers and perform sequence data analysis, nucleotide and protein sequences of the genes were obtained from sequence databases. The *Oryza sativa* homologs were used as queries to perform BLASTP analyses in Phytozome (https://phytozome.jgi.doe.gov/pz/portal.html), Plaza (https://bioinformatics.psb.ugent.be/plaza/versions/plaza_v4_5_monocots/) and NCBI (https://www.ncbi.nlm.nih.gov) databases. In addition, to perform *in silico* sequence

comparisons, the four newly sequenced bamboo genome obtained from *Bonia amplexicaulis*, *Guadua angustifolia*, *Olyra latifolia*, and *Raddia guianensis* were retrieved from bamboo genome database (https://gendbe.computational.bio.uni-giessen.de/cgi-bin/login.cgi). The set criteria for BLASTP analysis was as following- E-value threshold $\leq e^{-10}$, identity $\geq 40\%$ and length coverage $\geq 40\%$ of the query sequences. If no homologous sequences were identified within the set parameters, it is referred as 'no hit' (NH). In situations where multiple hits were obtained, only the best BLAST hit was considered for further study.

3.7. Selection of candidate *F-box* and *SRNase* genes based on *in silico* gene expression analysis

In order to understand the molecular regulation behind SRNase based GSI, the putative pistil candidate, *SRNase* and pollen candidate *Fbox* genes were selected. The *in silico* gene expression analyses were performed in BAR expression database (http://bar.utoronto.ca/). In case of *FBox* gene family, all 61-rice *FBox* genes having leucine (Leu) rich repeats (LRR) and their homologous *Brachypodium* genes were considered and their Reads Per Kilobase of transcript, per Million mapped reads (RPKM) were studied across available tissue stages (shoot, root, mature leaf, inflorescence, anther, stigma, ovary, embryo and endosperm). Rice and *Brachypodium* homologous *Fbox* genes having RPKM values >3000 and >100 respectively, were selected as biologically active, candidate *FBox* genes. On the other hand, in case of *SRNase* genes, all 8-rice *RNS* genes and their homologous *Brachypodium* genes were selected and their RPKM values were obtained across the above mentioned tissue stages. Here the minimum RPKM value cut off was set to 1000 and 90 for rice and *Brachypodium SRNase* genes respectively across all the tissues analysed. For both *FBox* and *SRNase,* genes, which were constitutively expressed across different tissue stages, were considered to be not involved in sexual incompatibility.

3.8. Isolation of genomic DNA

Genomic DNA isolation was performed from the leaf tissues using the DNeasy Plant Mini Kit (Qiagen, Germany). Approximately, 600mg samples were taken and treated with RNaseA (100mg/ml, Thermo Scientific, USA). The isolated genomic DNAs were finally eluted in 50 µl

DNase, RNase and protease free water and the quality was assessed using a BioSpectrometer (Eppendorf, Germany) and 1% low EEO agarose (SRL, India) gel electrophoresis.

3.9. Isolation of total RNA

Isolation total RNA from were performed by using a combination of TRIzol (Invitrogen, Carsbad, USA) and RNAeasy plant mini kit (Qiagen, Hilden, Germany; Das et al., 2010; 2016). To prevent any genomic DNA contamination, the lysate was treated with DNase I enzyme solution (Thermo Scientific, USA). Ultimately, evaluation of the quantity and quality of the isolated total RNA was performed in BioSpectrometer (Eppendorf, Germany) and agarose-formamide gel electrophoresis.

3.10. Preparation of cDNA libraries

The cDNA libraries were prepared using the Verso cDNA synthesis kit (Thermo Fisher Scientific) by following the procedure mentioned in the user manual. For the first strand cDNA synthesis, approximately 1µg of total RNA was used for each libraries to obtain maximum yield. The PCR cycle was 25ºC for 10 min, 42ºC for 1h and finally 70ºC for 15 min. After completion of the PCR, the synthesized cDNA products were visualized on agarose (SRL, India) gel electrophoresis.

3.11. Designing of PCR primers for gene amplification and expression analyses

The homologous genomic DNA and cDNA sequences obtained from related monocotyledonous species were multiple aligned in MUSCLE program (www.ebi.ac.uk/Tools/msa/muscle/). For the purpose of gene amplification, degenerate primers were designed from the conserved regions (Table X). Gene specific primers were designed from the isolated *B. tulda* gene sequences to perform gene expression analyses (**Table 3.1**). All the primers were designed in the Primer3 web tool (http://primer3.wi.mit.edu/).

Table 3.1. Summary of primers designed for gene amplification and real time RT-qPCR

Gene	Sequence (5'-3')	Primer length (bp)	GC content (%)	Tm (ºC)	Amplicon length (bp)	Experimental purpose

eIF4α	F-ATGCTCTCCCGTGGTTTCAAG	21	52.38	60.61	172	Gene expression analyses by real time RT-qPCR
	R-CAAGGGTAAGCTCATCTCTCTTCAC	25	48	60.91		
eEF1α	F-CGTGTGATTGAGAGGTTCGAGAA	23	47.83	60.61	211	
	R- AGGTACCCGTGATCATGTTCT	21	47.62	58.53		
CAC	F-TTTCTTCAGCAATGTGTGT	19	36.84	52.36	110	
	R-CCCTTTTGCTTGTTTCTTGA	20	40	58		
NTB	F-AGCTTATGGATATGTGGAGAA	21	38.10	54.02	111	
	R-CTTCTTGGCGTGATTGATACT	21	42.86	56.99		
CYP	F-TTTACGGCGCCAAGTTCG	18	55.56	63.17	100	
	R-TGGGAGCCGTTGGTGTTG	18	61.11	64.19		
UBQ5	F-ATCACGCTGGAGGTGGAGT	19	57.89	60.61	213	
	R-CTTCTTCTTGCGCTTCTTGGC	21	52.38	60.40		
UBQ3	F-GAAGGAGTCCACCCTGCACCT	21	61.90	63.81	136	
	R-CTGGATCTTGGCCTTCACGTTGTC	24	54.17	63.92		
18S	F-GCAACTATGGTGCCTCTATG	20	50	55.99	105	
	R-GCGACACCAGCAATCACCCA	20	60	63.36		
GAPDH	F-CCACAAACTGCCTTGCTCC	19	57.89	59.34	144	
	R-CCCTGCCACCCCTCCAG	17	76.47	61.45		
ACT7	F-GGATTGGTGGTTCTATTTTGGC	22	45.45	57.88	103	
	R-CACTTCATATGGACAATGCC	20	45	54.42		
RID1	F- CAAGAAGAAGAGGAGCCACCC	21	57.14	61.73	175	
	R- GAGCTTCCAGGGGAGGTTG	19	63.16	62.11		
SPL2	F- TTTCATTTGCTCGGGGAGTT	20	45	61.86	143	

	R- AATCTTGCCGCTCCGTGG	18	61.11	64.61		
MADS14	F-ATGGGGCGCGGGAAGGTG	18	72.22	74.8	762	PCR amplification of the target gene
	R-CTAGCCGTCGATGTGGCTCACCA	23	50	68.9		
	F-CTCGTGGAGAAGCAGAAGGTC	21	57.14	57.4	163	Gene expression analyses by real time RT-qPCR
	R-CTGCTGCGTCCTCTGCCCT	19	68.42	62.7		
MADS15	F-TCCATTTCCGAGCTGCAGAA	20	50	56.8	217	Gene expression analyses by real time RT-qPCR
	R-GTGGAAGAAGTGCCTGCTGAT	21	52.38	57.6		
MADS18	F-CACCAAGCACATCATCACCAACACC	25	52	60.5	102	
	R-GAGCTTCTGACTCCCCTGATCCTCT	25	56	61.3		
RNS1	F- CCCAACTACGCCAAGTGC	18	61.11	60.26	133	Gene expression analyses by real time RT-qPCR
	R- CGAGGTCCTTGATCTCCC	18	61.11	58.07		
	F- TGCAGTGGCCTGGCTCGT	18	66.68	66.67	625	PCR amplification of the target gene
	R- GGAACAGCTGCGTCTCGCC	19	66.82	68.42		
RNS2	F- GAGTTCGACTACTTCGCG	18	55.56	54.51	189	Gene expression analyses by real time RT-qPCR
	R- GTCCATGTCGAAGTTGGT	18	50	53.98		
RNS3	F- GTCTTACTGCGACACGAAGCAGA	23	52.17	64.08	132	
	R- TCGCTGTCGGGGTTGCAGTT	20	60	68.49		
RNS6	F- ATGCTGCACACCCACCACA	19	57.89	64.81	168	
	R-TCAGGGTAACCACATGTTCCATGAG	25	48	66.24		
Fbox4	F-GCAGGAGCTCAAGCTCGA	18	61.11	57.5	164	

	R- CCAGAACAGAATTGTTGACAGC	22	45.45	54.4		
Fbox7	F-CGGGTACTACGGGAAGGCT	19	63.16	58.8	225	
	R- GCATCCGTCATGTGTCCA	18	55.56	55.2		
Fbox19	F- GCGTGCTCGCTCACCTG	17	70.59	59.6	173	
	R- AGGCTGTCCGTCCCGGA	17	70.59	61.8		
Fbox22	F- GTGGTGCAATGCCAACCTT	19	52.63	56.7	191	
	R- TAAGTTATTCATGTTCTGCTGGCAC	25	40	55.6		
Fbox28	F- CATCCTTCCACGACCTCTCCAT	22	54.55	58.5	189	
	R- AGCCCCAGCAGCTTCGTGA	19	63.16	62.1		
Fbox30	F- GGCTCCTTCCATTGATCAAGA	21	47.62	54.7	145	
	R- CTGCAACCTTCGAGACTGAG	20	55	55.4		
Fbox52	F- GGAGACAGGGATCTGCAGAGTAAA	24	50	58	219	
	R- CCCTGGGTTCTCATTAACACCA	22	50	56.8		

3.12. PCR amplification of targeted homologous genes

The PCR amplification was performed using Go Taq DNA Polymerase (Promega, USA) in S1000 PCR detection system. The reagents in the final PCR master mix were 5X buffer for Taq polymerase, 1.5-3 mM $MgCl_2$, 0.2 mM dNTPs, 0.25 µM forward and 0.25 µM reverse primers, 2 µL normalized cDNA/20 µL PCR master mix and 1.25U/50 µL Taq polymerase. The PCR amplification cycle was: initial denaturation at 95ºC for 3 minutes, followed by 25-30 cycles of 94ºC for 30 seconds, 55ºC for 55 seconds, 72ºC for 30-45 seconds for 1 kb of PCR product, final extension at 72ºC for 1 minutes, followed by 4ºC forever.

3.13. Gel elution of the amplified PCR products

Amplified PCR products were gel eluted using GeneJET gel elution kit (Thermo Scientific, USA). Approximately 150-180 µL of PCR products were loaded on agarose (SRL, India) gel stained with 10mg/ml ethidium bromide ($C_{21}H_{20}BrN_3$, SRL, India). Bands of desired molecular weight was dissolved into equal volume of binding buffer by incubating at 55-60ºC. After subsequent washing and passing through DNA binding column, PCR products were eluted in 20µl nuclease free water.

3.14. Preparation of calcium competent *E. coli* Dh5α cells

Calcium competent *E. coli* Dh5α cells were prepared for cloning and transformation experiments. At first, *E. coli* Dh5α cells were grown overnight to be used as a starter culture, was inoculated in 250 ml of liquid LB medium and grown at 37 °C. The density of cells was measured at 600 nm in a BioSpectrometer at repeated intervals (Eppendorf, Germany) at 600 nm. When density of cells reached at 0.35-0.40, cells were pelleted by successive centrifugations and were further treated with chilled solution of 100mM $MgCl_2$, 100mM $CaCl_2$ and 85mM $CaCl_2$ in 15% glycerol. Finally, the pellet was dissolved in 85mM $CaCl_2$ in 15% glycerol, snap frozen in liquid nitrogen and stored at -80 °C refrigerator.

3.15. Cloning of amplified PCR products and transformation

The amplified PCR products having 3’ overhang were cloned using TA based pGEM®-T Easy vector systems (Promega, USA), whereas the blunt end products were cloned using CloneJET PCR Cloning Kit (Thermo Fisher Scientific, USA). In both the procedures, PCR products were ligated with approximately 25ng of vector DNA in 3:1 ratio. The transformed cells were plated on solid LB media with 1.6% agar and Ampicillin (100µg/ml), which was used as a selectable marker. For the purpose of selection in TA cloning, X gal (20mg/ml, dissolved in formamide) and Isopropyl β- d-1-thiogalactopyranoside (IPTG; 0.1M) were also added to the solid media. Finally, plates were incubated at 37ºC overnight to promote bacterial growth.

3.16. Colony PCR to select putatively transformed colonies

Ten to twenty, randomly selected, single colonies were added to individual PCR tubes containing PCR master mixture to carry out. PCR. The composition of both master mix and PCR cycling conditions were similar as mentioned in section 3.12.

3.17. Isolation of plasmids, Sanger's sequencing, assembly

The putatively transformed colonies were grown overnight at 37º C in 10 ml liquid LB medium having Ampicillin (100µg/ml) as selectable marker. Plasmids were isolated using GeneJET Miniprep kit (Thermo Scientific, USA). The bacterial pellets obtained from overnight grown culture were resuspended following cell lysis and neutralization using the corresponding buffers provided with the kit. The plasmids were finally eluted using 30µl of nuclease free water and were sent for sequencing by using Sanger's method (AgriGenome, http://www.aggenome.com/). Both gene and vector specific primers were used to sequence the plasmids. The trimmed nucleotide sequences were assembled in the CAP3 program (http://doua.prabi.fr/ software/cap3) to obtain assembled contigs and were finally deposited to NCBI database (http://www.ncbi. nlm.nih.gov/).

3.18. *In silico* characterization of amino acid sequences

In order to perform *in silico* analysis of the predicted amino acids, the obtained sequences were multiple aligned using MUSCLE program (www.ebi.ac.uk/Tools/msa/muscle/) and the functional domains and biologically important residues were identified. In addition, the molecular weight, isoelectric point, total number of negatively and positively charged residues, aliphatic index, instability index and grand average of hydropathicity (GRAVY) were also calculated using the tools available in ExPASy database (https://www.expasy.org/).

3.19. Phylogenetic analysis

In order to perform phylogenetic analysis, the full-length amino acid sequences were multiple aligned in MUSCLE tool (www.ebi.ac.uk/Tools/msa/muscle/) along with homologous sequences from related monocot species. A Neighbour Joining (NJ) tree was further constructed using the MEGA7 software and its default set parameters (Kumar et al., 2016). For all the phylogenetic analyses, bootstrap values used were1000.

3.20. Real time RT-qPCR analyses

Real time RT-qPCR analyses was performed by using SsoAdvanced™ Universal SYBR® Green Supermix (BioRad, USA) in a CFX connect real-time PCR detection system (Bio Rad, USA). The volume of the PCR mixture used was 10 µL, which contained 1X concentration of supermix buffer, 0.25 µM for each forward and reverse primers and 2 µL of cDNA (1/20th diluted from the stock). The PCR amplification cycles used were: 95 ºC for 30 s, followed by 40 cycles of 94 ºC for 10 s and at 55 ºC for 40 s. A dissociation curve (65–95ºC, at increments of 0.5ºC) analysis was performed to ensure absence of any primer-dimer. The relative changes in gene expression were calculated using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen 2001). The reference genes *eIF4α* and *UBQ5* were used for normalization of gene expression data for *AP1* like genes and *RNS* genes respectively.

3.21. Statistical analyses to determine the stability of reference genes

In order to identify the most suitable reference gene in the studied experimental set up, two different programmes geNorm (https://genorm.cmgg.be/, Vandesompele et al. 2002), NormFinder (https://moma.dk/normfinder-software, Andersen et al. 2004) along with the comprehensive ranking analysis tool, RefFinder (http://150.216.56.64/ referencegene.php, Silver et al. 2006) were used. In addition to this, the optimum number of reference genes required for a particular experimental data set was determined using the pairwise variation analysis ($V_{n/n+1}$), which was available in the geNorm program.

3.22. *In silico* prediction of cis elements in the promoter regions

In order to identify important cis elements present in the promoter regions, approximately 1500 bp upstream regions from translation initiation site (ATG) were analysed in Plant CARE database (http://bioinformatics.psb.ugent.be/webtools/plantcare /html/) and new PLACE database (https://www.dna.affrc.go.jp/PLACE/?action=newplace). In order to conduct dendrogram analyses, the data obtained from Plant Care database were used as input data in DendroUPGMA program (http://genomes.urv.cat/UPGMA/index.php?entrada=Example1).

3.23. *In silico* identification of motifs

Motifs present in the predicted proteins of *B. tulda*, and other homologs were identified using Multiple EM for Motif Elicitation (MEME) version 5.3.0. tool. The parameters, which were used for this analysis were number of repetitions: any, maximum number of motifs: 20, maximum width: ≤200 and minimum width: ≥6.

4. Results

Objective I: Identification of stable reference genes in bamboo suitable for gene expression analyses

4.1. Expression profiling of the candidate reference genes in diverse organs, developmental stages and among different species

Gene expression profiling of the ten candidate genes (*eukaryotic initiation factor 4α: eIF4α, eukaryotic elongation factor 1α: eEF1α, clathirin adaptor complexes medium subunit: CAC, nucleotide tract-binding protein: NTB, peptidyl prolyl cis-trans isomerase/cyclophilin: CYP, ubiquitin 5: UBQ5, UBQ3, 18S RIBOSOMAL RNA: 18S, glyceraldehyde-3-phosphate dehydrogenase: GAPDH* and *actin: ACT7*) were performed using a SYBR green dye based detection system. The Ct values ranged from 17-26 cycles, whereas for most of the genes the Ct values fall between 22-24 cycles. Out of the ten candidate genes, the highest Ct value was observed for *ACT7* (29.55 ± 0.03), whereas it was lowest in case of *UBQ3* (16.14 ± 0.24, **Figure 4.1**).

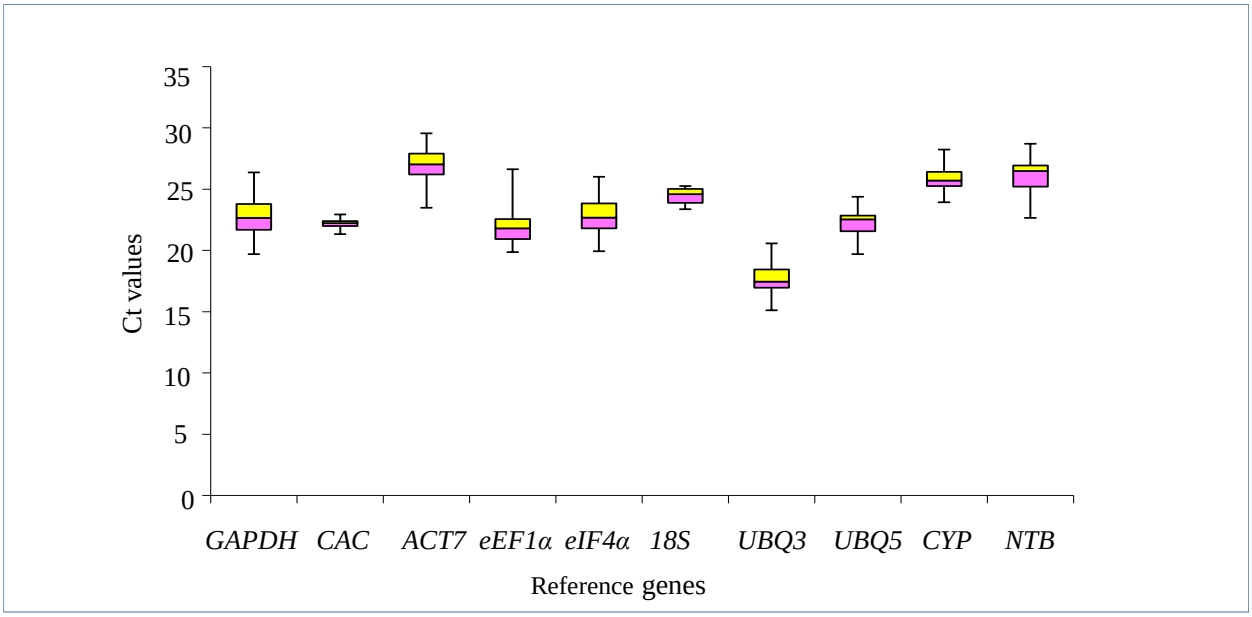

Figure 4.1. Distribution of Ct values of the ten candidate reference genes across different experimental conditions. The 25th and 75th percentile is indicated in each box. Median is depicted by the straight line crossing the box. The whisker caps represent the maximum and minimum values. Abbreviations used- Ct- threshold cycle, gene names are same as mentioned in Section 4.1

4.2. *In silico* study to analyze the stability of the studied reference genes in various experimental conditions

To assess stability of the ten, selected reference genes' expression, three different programmes, which were geNorm (Vandesompele et al. 2002), NormFinder (Andersen et al. 2004) and RefFinder (Silver et al. 2006) were used.

4.2.1. Evaluation of gene expression stability using geNorm

The geNorm is a Visual Basic application (VBA) tool implemented in Microsoft excel, where the two most stable reference genes are identified based on their expression ratio across all the samples analyzed (Vandesompele et al., 2002). The average pairwise variation among all the selected genes are calculated and is represented as the stability value (M). Gene pairs with lower

M value is considered to be more stable than those with higher M value in the different experimental conditions (**Figure. 4.2**).

4.2.2. Evaluation of gene expression stability using NormFinder

In addition to the geNorm, expression stability of candidate reference genes were also analysed by another VBA tool NormFinder (Andersen et al., 2004). NormFinder predicts candidates based on their expression stability under a particular experimental condition. Genes having higher stability values are considered less stable than those having lower values in different experimental conditions (**Table 4.1**).

4.2.3. Evaluation of gene expression stability using RefFinder

In addition to the two above-mentioned programmes, another web based computational tool, RefFinder was used to obtain a comprehensive ranking of candidate reference genes based on their geometric mean (http://150.216.56.64/ referencegene.php, **Table 4.2**).

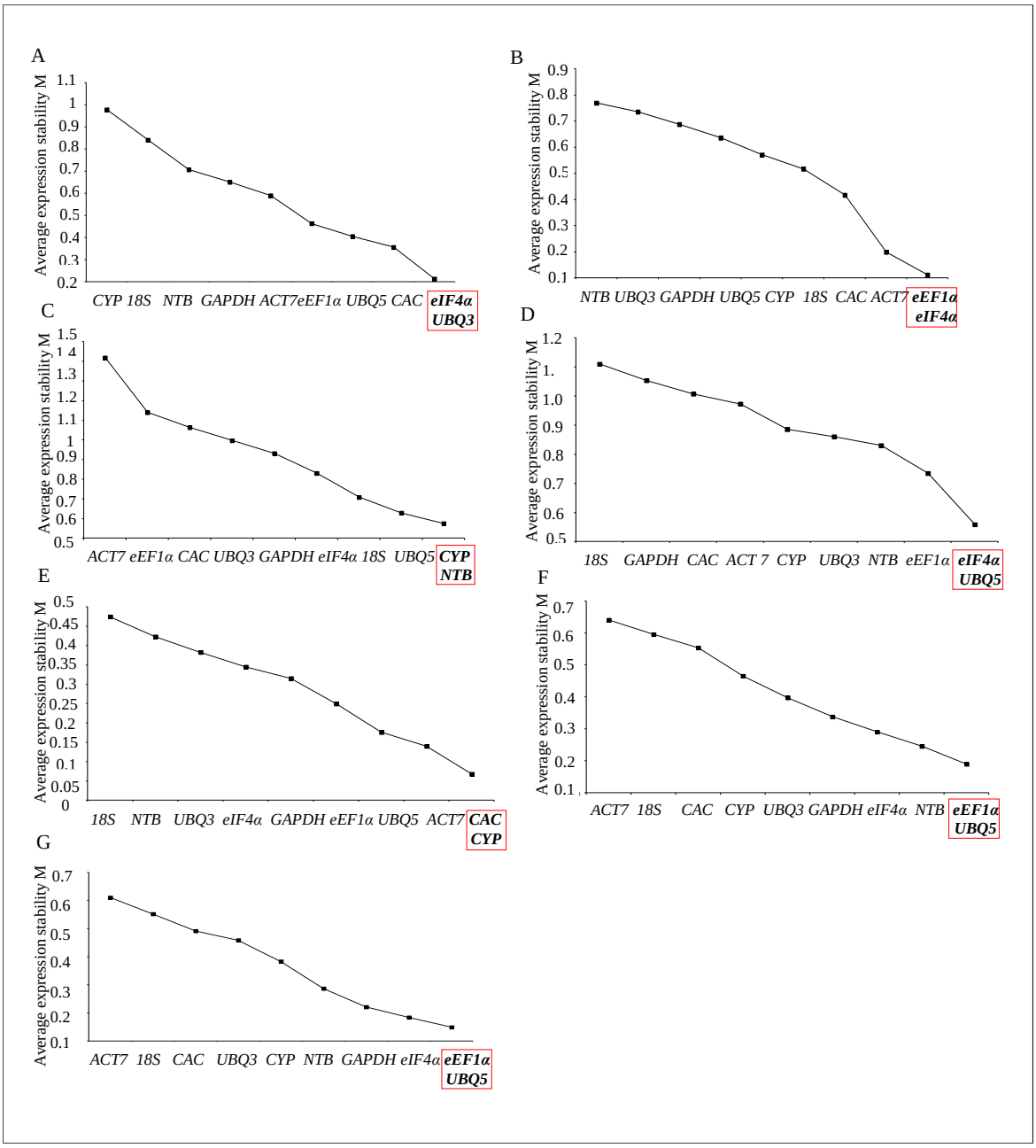
A
Average expression stability M
CYP 18S NTB GAPDH ACT7eEF1α UBQ5 CAC eIF4α UBQ3
B
Average expression stability M
NTB UBQ3 GAPDH UBQ5 CYP 18S CAC ACT7 eEF1α eIF4α
C
Average expression stability M
ACT7 eEF1α CAC UBQ3 GAPDH eIF4α 18S UBQ5 CYP NTB
D
Average expression stability M
18S GAPDH CAC ACT 7 CYP UBQ3 NTB eEF1α eIF4α UBQ5
E
Average expression stability M
18S NTB UBQ3 eIF4α GAPDH eEF1α UBQ5 ACT7 CAC CYP
F
Average expression stability M
ACT7 18S CAC CYP UBQ3 GAPDH eIF4α NTB eEF1α UBQ5
G
Average expression stability M
ACT7 18S CAC UBQ3 CYP NTB GAPDH eIF4α eEF1α UBQ5

Figure. 4.2. Gene expression stability and ranking of the selected, ten, candidate reference genes as predicted by geNorm software. (A) Leaf tissues across different species, (B) internodes obtained from different species, (C) rhizome obtained from different species, (D) nine different tissues from six different organs, three developmental regions of (E) leaf, (F) internodes collected from top, middle and base of a branch, (G) three developmental stages of inflorescence buds (early, middle and late). Abbreviations are same as mentioned in Section 4.1

Table 4.1. Predicted stability rankings of ten candidate reference genes using expression data obtained from four species, nine organs and three developmental stages of tropical bamboos. The values within brackets indicate order of ranking of the candidate reference genes predicted by NormFinder analysis.

Species		***B. tulda, B. balcooa, B. bambos, B. vulgaris***			***B. tulda***	***B. tulda***		
Experimental sets		Leaf	Internode	Rhizome	Young leaves from flowering and non flowering culms, flag leaf, possible flag leaf, culm sheath, internode, root, rhizome, early staged inflorescence bud	Leaf (tip, middle, base)	Internode (tip, middle, base)	Inflorescence bud (early, middle, late)
Reference genes	***ACT7***	0.77 (6)	0.61 (6)	2.41 (10)	0.93 (7)	**0.02 (1)**	0.69 (10)	0.81 (10)
	GAPDH	0.92 (7)	0.68 (8)	0.54 (2)	1.12 (9)	0.34 (6)	0.51 (6)	0.61 (8)
	UBQ3	0.37 (4)	0.71 (9)	0.81 (5)	0.65 (4)	0.56 (9)	0.53 (7)	0.27 93)
	UBQ5	0.10 (2)	0.46 (4)	0.59 (3)	0.63 (3)	0.06 (2)	0.46 (4)	0.26 (2)
	18S	1.07 (9)	0.67 (7)	0.98 (7)	1.31 (10)	0.65 (10)	0.64 (9)	0.63 (9)
	CAC	**0.10 (1)**	**0.13 (1)**	1.07 (8)	0.89 (6)	0.26 (4)	0.61 (8)	0.32 (4)
	CYP	1.45 (10)	0.59 (5)	0.75 (4)	0.54 (2)	0.30 (5)	0.46 (5)	**0.13 (1)**
	NTB	0.96 (8)	0.77 (10)	**0.28 (1)**	0.80 (5)	0.45 (8)	0.25 (2)	0.56 (7)
	eIF4α	0.30 (3)	0.33 (2)	0.94 (6)	**0.54 (1)**	0.40 (7)	**0.14 (1)**	0.44 (6)
	eEF1α	0.47 (5)	0.44 (3)	1.36 (9)	0.95 (8)	0.18 (3)	0.34 (3)	0.44 (6)

Table 4.2. Predicted stability ranking of ten candidate, reference genes using expression data obtained from four species, nine organs and three developmental stages of tropical bamboos. Values within brackets indicate order of rankings of the candidate reference genes in RefFinder analysis.

Species	***B. tulda, B. balcooa, B. bambos, B. vulgaris***	***B. tulda***	***B. tulda***

Experimental sets		Leaf	Internode	Rhizome	Young leaves from flowering and non flowering culms, flag leaf, possible flag leaf, culm sheath, internode, root, rhizome, early staged inflorescence bud	Leaf (tip, middle, base)	Internode (tip, middle, base)	Inflorescence bud (early, middle, late)
Reference genes	***ACT7***	6.00 (6)	5.33 (5)	10.00 (10)	5.24 (5)	**2.21 (1)**	8.80 (10)	7.40 (10)
	GAPDH	7.65 (7)	5.66 (6)	3.83 (4)	9.00 (10)	6.45 (7)	6.22 (8)	6.70 (8)
	UBQ3	2.99 (4)	7.77 (9)	6.12 (8)	4.86 (4)	8.97 (10)	6.96 (9)	3.98 (4)
	UBQ5	2.00 (2)	4.28 (4)	2.91 (3)	2.21 (2)	2.51 (2)	3.46 (4)	**1.86 (1)**
	18S	**1.57 (1)**	**1.41 (1)**	4.76 (5)	5.29 (6)	2.63 (3)	5.66 (7)	4.00 (5)
	CAC	9.74 (10)	5.73 (7)	2.38 (2)	4.05 (3)	2.66 (4)	5.01 (5)	2.78 (2)
	CYP	8.00 (8)	9.15 (10)	**1.50 (1)**	5.32 (7)	7.67 (9)	2.78 (2)	7. 27 (9)
	NTB	2.45 (3)	2.00 (2)	5.96 (7)	**1.41 (1)**	7.45 (8)	**2.00 (1)**	5.42 (7)
	eIF4α	4.40 (5)	2.91 (3)	9.00 (9)	6.62 (9)	4.21 (5)	2.82 (3)	3.64 (3)
	eEF1α	6.00 (6)	5.33 (5)	10.00 (10)	5.24 (5)	2.21 (1)	8.80 (10)	7.40 (10)

4.3. Validation of the stability of selected reference genes using two flower associated target genes

In order to validate the utility of identified reference genes, the two most stable (*eIF4α, UBQ5*) and one least stable (*18S*) genes were selected for normalization of gene expression data obtained from seven different vegetative and reproductive plant organs of *B. tulda*.

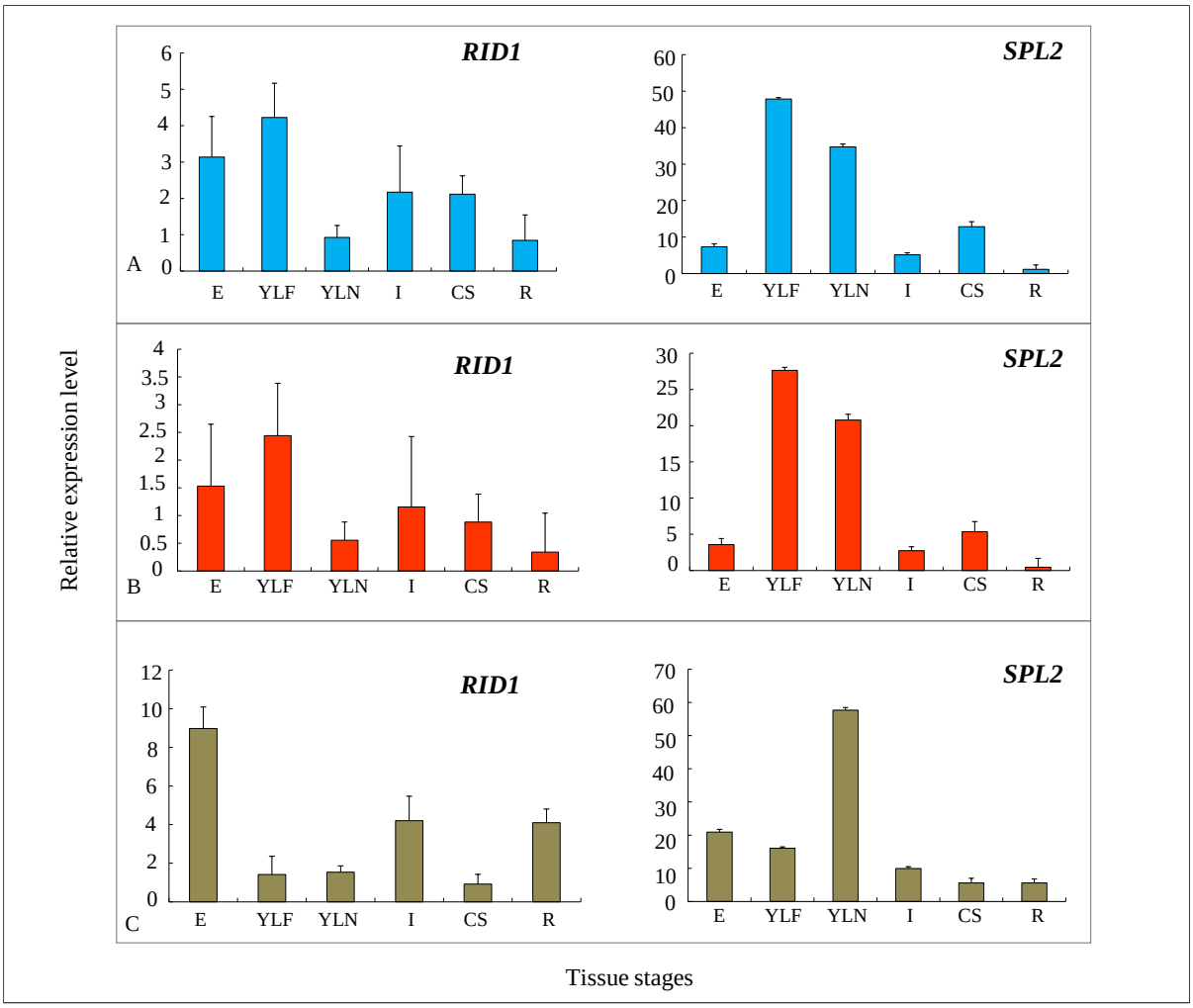

Figure 4.2 Relative quantification of floral inductive transcription factor genes *RID1* and *SPL2* by using the two most stable *eIF4α* (A) and *UBQ5* (B) and one least stable reference gene *18S* (C) in different organs of *B. tulda*. The relative fold change was calculated by $2^{-\Delta\Delta Ct}$ method using the rhizome tissue as calibrator. Each bar represents mean of three biological replicates ± SE. Abbreviations used: E- early staged inflorescence bud, YLF- young leaves from flowering culm, YLN- young leaves from non flowering culm, I- internode, CS- culm sheath, R- root.

The tissue specific relative gene expression pattern of two target genes associated with floral inductive functions, *RID1* and *SPL2* were analyzed across various plant organs using the selected reference genes (**Figure 4.2**). When the *eIF4α* and *UBQ5* genes were used for data normalization, transcript abundance of both the target genes were higher in young leaves from

flowering culm (YLF) than that of a non-flowering culm (YLN; **Figures 4.2A, B**). However, when *18S* was selected to normalize the expression data, due to its low stability expression patterns were altered (**Figure 4.2C**). In case of *RID1* gene, there was almost no difference in gene expression in the YLF and YLN tissues; whereas for *SPL2*, the gene expression was higher in YLN compared to YLF.

Objective II: A comparison between spikelet vs pseudospikelet and investigations to understand the development of anthers, ovary and setting of seeds in the inflorescences of *B. tulda* Roxb.

4.4. Study on the frequency of spikelet vs. pseudospikelet in an entire flowering cycle of *B. tulda*

The inflorescence structure of Poaceae grasses is known as spikelet. However, in addition to the true spikelet, bamboos also bear another inflorescence known as pseudospikelet (**Figure 4.3**).

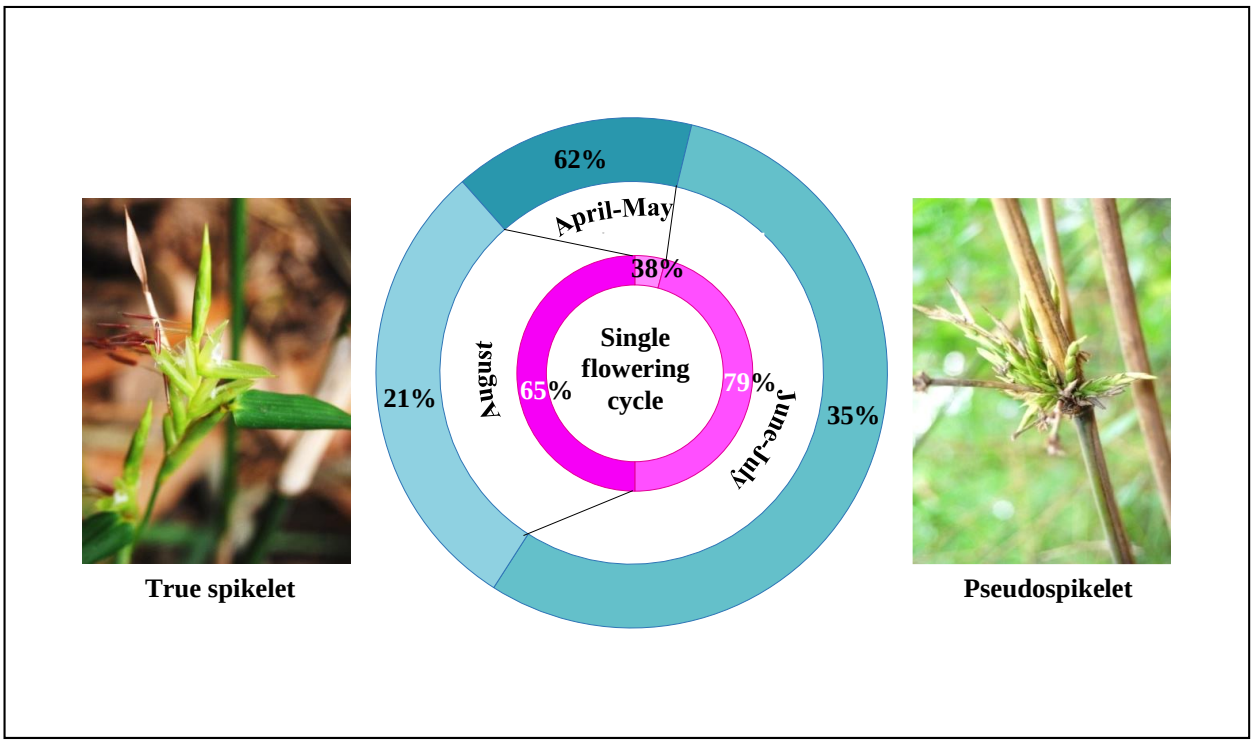

Figure 4.3. Schematic representation depicting the comparative frequency of true spikelet and pseudospikelet during an entire flowering cycle of *B. tulda*. Pink circle indicates

frequency of pesudospikelet in a flowering cycle, whereas blue circle indicates frequency of true spikelet. The rate of occurrence is mentioned with in the circles

In order to understand if there is any specific pattern of development of true vs. pseudospikelet, total number of true and pseudospikelet was counted within a particular flowering cycle (**Figure 4.3**). During the onset of flowering, the number of true spikelet was higher compared to pseudospikelet. As the flowering cycle continued, both types of spikelets coexisted throughout the entire phase. However, the frequency of true spikelet gradually declined during the final months of reproductive phase and pseudospikelet increased (**Figure 4.3**). During the onset of summer (April to May), the number of true spikelets increased and simultaneously pseudospikelets started emerging as well. The pseudospikelets attained maximum number during July when they arose from the nodes of flowering branches (**Figure 4.3**). However, number of both true spikelets and pseudospikelets decreased by August and started withering by October.

4.5. Study on the developmental progression of vegetative and inflorescence buds of *B. tulda* by scanning electron microscopy

In order to understand the morphological progression of vegetative and floral buds of *B. tulda*, SEM analysis was performed (**Figure 4.4**). The vegetative bud was ~7 mm in length (**Figure 4.4A**). SEM analysis demonstrated that the vegetative bud is triangular in shape (**Figure 4.4B**). Initiation of inflorescence meristem began after the transition from vegetative SAM to IM. The early staged inflorescence bud was ~4 mm long and morphologically very similar to the vegetative bud (**Figure 4.4C**). However, in contrary to vegetative bud, here, bulging of the meristem tip is observed, which ultimately is converted to the inflorescence meristem (**Figure 4.4D**). The middle staged bud is ~9 mm in length (**Figure 4.4E**). At this stage, differentiation of floral primordia began at the spikelet base (**Figure 4.4F**). In addition to this, initiation of the outer whorl organ (lemma and palea) was also observed. However, the apical part of the SM remains undifferentiated at this stage. In contrast to true spikelet, multiple inflorescence primordia was observed in the pseudospikelet bud (**Figure 4.4H**).

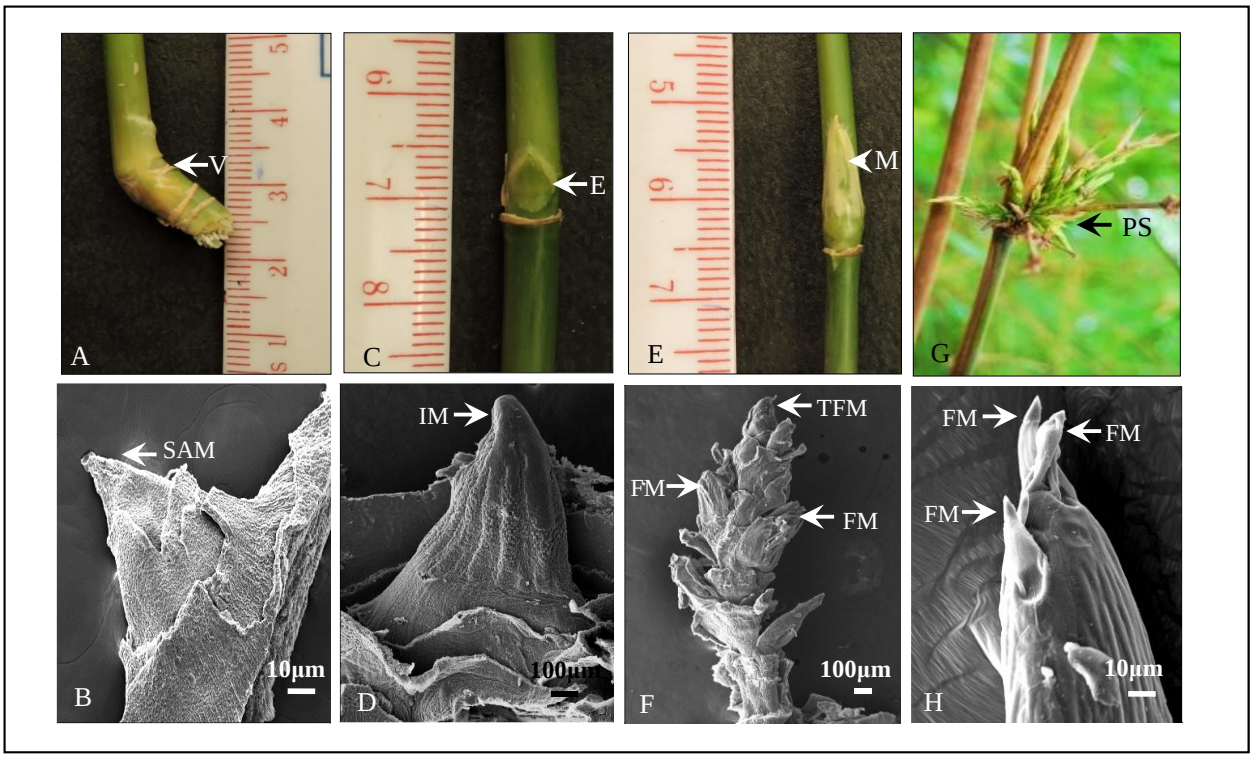

Figure 4.4. Morphological observations of vegetative and inflorescence buds of *B. tulda*. (A) External morphology of vegetative bud; (B) SEM observation of vegetative bud; (C) External morphology of early staged inflorescence bud; (D) SEM observation of early staged inflorescence bud showing initiation of inflorescence meristem; (E) External morphology of middle staged inflorescence bud; (F) SEM observation of middle staged inflorescence bud showing development of spikelet meristem; (G) External morphology of pseudospikelet; (H) SEM observation of pseudospikelet meristem showing inflorescence buds from different developmental stages; Abbreviations used- V- vegetative bud, E- early bud, M- middle staged bud, PS- pseudospikelet, SAM- shoot apical meristem, IM- inflorescence meristem

4.6. Comparison of frequency of seeds produced by spikelet and pseudospikelet of *B. tulda*

The presence of two different types of inflorescence is an important reproductive feature of bamboo. In order to understand the biological significance and its impact on overall reproductive success, comparison of total number seeds produced by spikelet and pseudospikelet inflorescences were counted and compared (**Figure 4.5**).

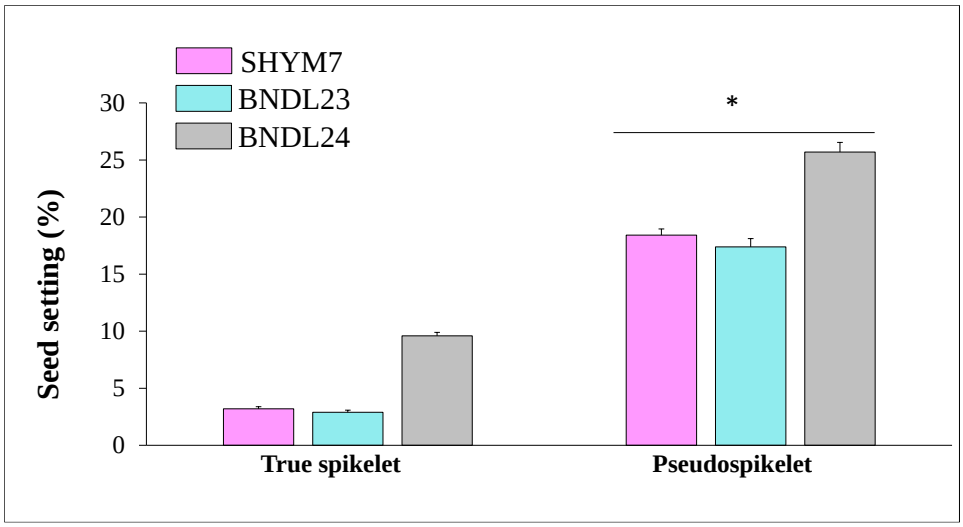

Figure 4.5 Comparison of the rate of seed settings between true spikelet and pseudospikelet of *B. tulda*. Each bar represents the rate of seed settings ±SD.

With respect to all the three populations, the rate of seed setting by pseudospikelet was significantly higher (p-value= 0.0000, 17.3-25.7%) than true spikelet (3.2-9.6%; **Figure 4.5**)

Objective III: Study on the pollen-pistil interactions and identification of genes regulating this process in bamboo

4.7. *In vivo* self and cross pollination analyses to study nature of genetic compatibility in *B. tulda*

In order to understand the nature of genetic interaction between pollen and pistil, *in vivo* pollen-pistil interaction was studied in two distant populations of *B. tulda* (**Figure 4.6A, C**). Staining of the pollinated stigmas resulted the attached pollen grains to obtain blue colour, while the germinated pollen tubes were hyaline (**Figure 4.7A, B**). In case of self pollination, 62 out of 834 pollens were germinated, whereas, the number was 300 out of 802 pollens for cross pollination. Therefore, the percentage of pollen germination was significantly higher ($p < 0.000$) in case of cross pollination (32.9-41.3 %) than self pollination (4.2-8.6 %; **Figure 4.7 C**).

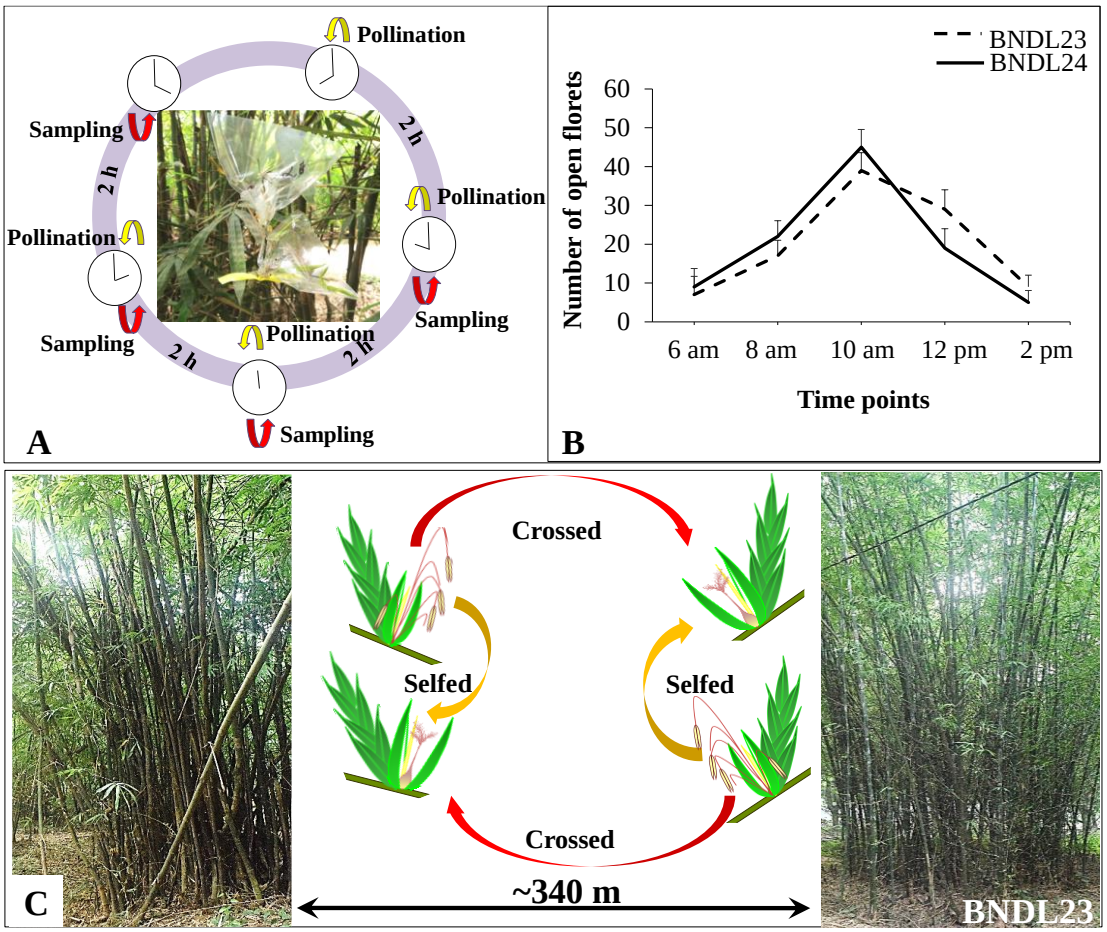

Figure 4.6. Experimental design to study *in vivo* pollen pistil interaction in *B. tulda*. (A) Time course experiment conducted to identify optimum time point for pollination. (B) Line graph demonstrating optimum time point for opening of *B. tulda* flower. (C) Schematic representation of the experimental set up. Abbreviations used: h- Hour

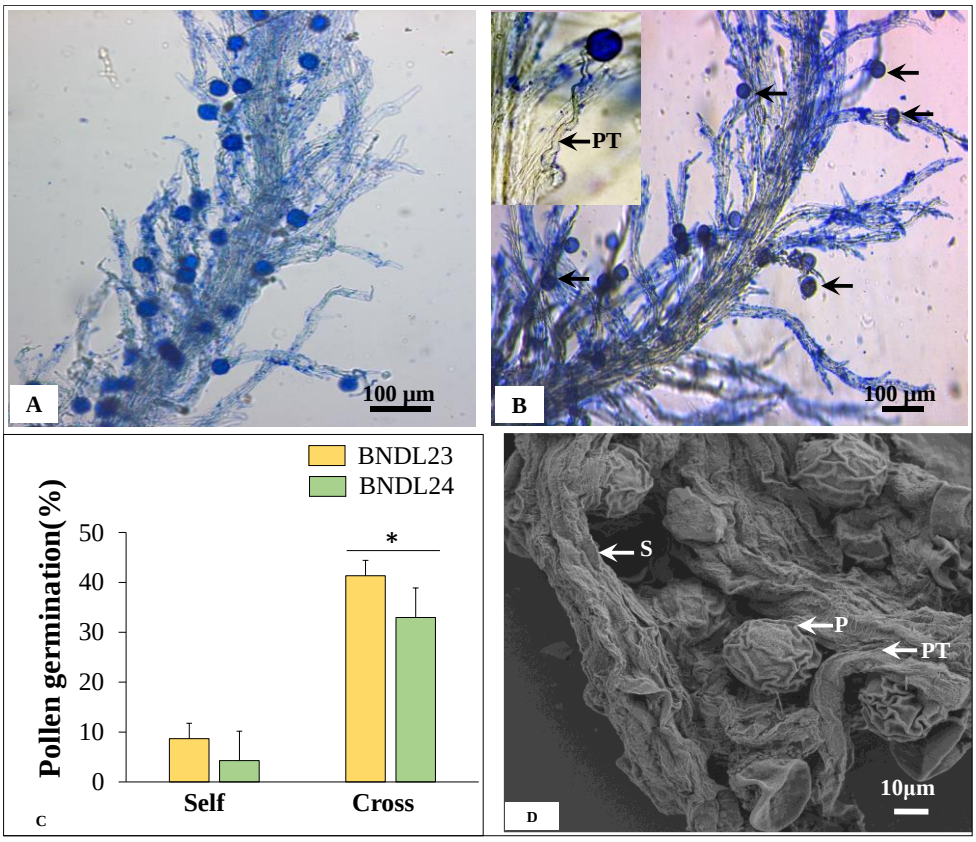

Figure 4.7. Microscopic observation to study *in vivo* pollen pistil interaction in *B. tulda*. (A) Magnified view of stained pollinated stigma after self pollination. (B) Magnified view of stained pollinated stigma after cross pollination. Pollen tubes are marked by arrow. (C) Histogram demonstrating relative abundance of selfed vs. crossed pollination in two population of *B. tulda*. (D) Scanning electron microscopic (SEM) image of stigma after cross pollination. Abbreviations used: S- Stigma, PT- Pollen tube, P- Pollen.

4.8. PCR isolation, sequencing and molecular characterization of *S-RNase* and *FBox* genes from *B. tulda*

4.8.1. *In silico* identification of RNase T2 family genes from all the sequenced genomes of monocotyledonous plants

In silico sequence querry using eight rice RNase T2 genes, could not identify all the homologous genes in majority Poaceae as well as non-Poaceae plants (**Table 4.3**). BLAST analysis suggested reduction of *RNS* gene homologs in bamboo genomes. Out of eight *RNS* genes from rice, only

six could be identified in *R. guinensis* and *G. angustifolia*, five in *B. amplexicaulis* and *O. latifolia*, and only four in *P. edulis* (**Table 4.3**). In majority of the Poaceae members, *RNS7* and *RNS8* genes were absent; however, *RNS7* orthologs were present in *R. guinensis* and *G. angustifolia* genomes.

Table 4.3: *In silico* identification of homologous RNase/T2 genes from bamboos. BLASTP analysis was performed where *O. sativa* sequences were used as query. For any individual gene if more than one BLAST hits were obtained, the top BLAST hit was selected for further analyses. Overlapping homologous sequences between different genes are marked with their homologous gene number in superscript.

***OsRNS* genes** / **Bamboo species**	***RNS1* (Os07g 43670)**	***RNS2* (Os01g 67180)**	***RNS3* (Os08g 33710)**	***RNS4* (Os09g 36680)**	***RNS5* (Os09g 36700)**	***RNS6* (Os01g 67190)**	***RNS7* (Os07g 43600)**	***RNS8* (Os07g 43640)**
Phyllostachys edulis	*PH0100 0064G1 820*[1]	*PH0100 0244G0 580*[2]	*PH0100 0729G0 220*[3]	*PH0100 6208G0 020*	*PH0100 0729G0 220*[3]	*PH0100 0244G0 580*[2]	*PH0100 0064G1 820*[1]	*PH0100 0064G1 820*[1]
Raddia guinensis	*Rgu023 360.1*	*Rgu009 565.1*	*Rgu006 396.1*	*Rgu016 984.1*	*Rgu009 077.1*	*Rgu009 565.1*	*Rgu010 963.1*	*Rgu023 360.1*
Bonia amplexicaulis	*Bam002 260.1*	*Bam030 490.1*	*Bam032 233.1*	*Bam039 353.1*	*Bam038 486.1*	*Bam030 490.1*	*Bam002 260.1*	*Bam002 260.1*
Guadua angustifolia	*Gan015 904.1*	*Gan011 954.1*	*Gan009 694.1*	*Gan012 298.1*	*Gan012 298.1*	*Gan010 206.1*	*Gan025 662.1*	*Gan009 694.1*
Olyra latifolia	*Ola004 968.1*	*Ola038 502.1*	*Ola013 475.1*	*Ola037 740.1*	*Ola037 737.1*	*Ola038 501.1*	*Ola004 968.1*	*Ola004 968.1*

4.8.2. Selection of candidate *SRNase* and *FBox* genes involved in the pollen-pistil interaction

In order to select the candidate *SRNase* and *FBox* genes involved in compatibility response in pistil and anther respectively, *in silico* expression analysis of the entire RNase T2 and *Fbox* genes with Leu repeats were done in rice and *Brachypodium*. The cut-off value of minimum

RPKM for both *SRNase* and *FBox* genes are mentioned in section 3.7. In case of *SRNase* genes, the RPKM values of five rice RNase T2 family genes and three homologous *Brachypodium* genes were observed above the set criteria (**Figure 4.8**). Among them, the *OsRNS1* and *OsRNS4* were primarily expressed in root and shoot tissues, and *OsRNS3* in the leaves respectively (**Figure 4.8A**). However, since the homologous *BdRNS1* and *BdRNS3* genes were highly expressed in pistil, they were selected to study its expression in *B. tulda* (**Figure 4.8B**). *OsRNS4* was not selected due to its very low expression in pistil and the *OsRNS6* having RPKM values below the set criteria was selected as a negative control (**Figure 4.8A**). Similarly, for *Fbox* genes, the RPKM values of rice and *Brachypodium* homologs were analysed (**Figure 4.9)**.

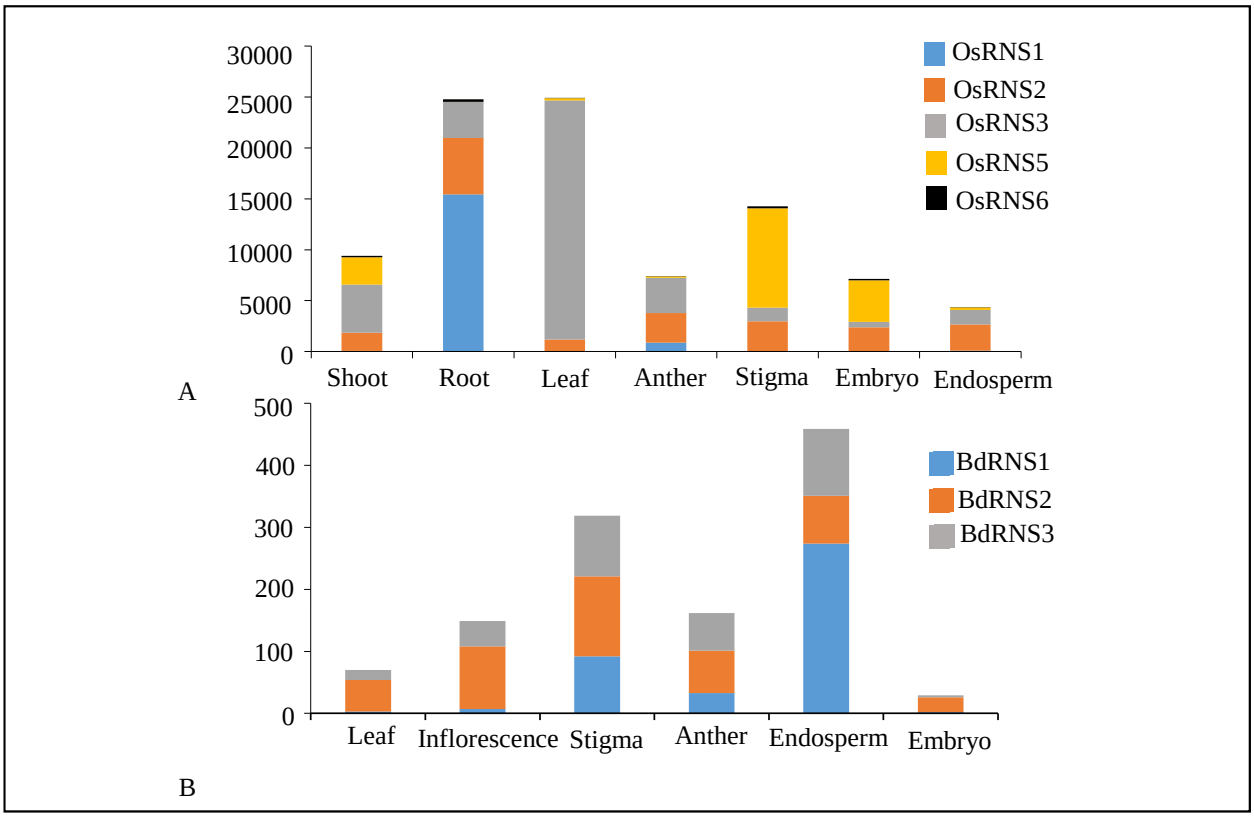

Figure 4.8. *In silico* gene expression analysis of *RNS* genes from (A) rice and (B) *Brachypodium*. Selection of these genes were performed based on their RPKM assessed expression level across different vegetative and reproductive tissue stages. The X axis denotes tissue stages and the Y axis indicates RPKM values.

Both in rice and *Brachypodium*, six *FBox* genes demonstrated RPKM values above the set criteria (**Figure 4.9**). However, the *BdFB23* were not included in further analysis due to its very low expression in anther. Overall, this analysis selected four candidate *SRNase* (*RNS1, RNS2, RNS3* and *RNS6*) and seven candidate *F Box* (*FBL4, FBL7, FBL19, FBL22, FBL28, FBL30* and *FBL52*) genes to study expression of their homologous gene in *B. tulda*.

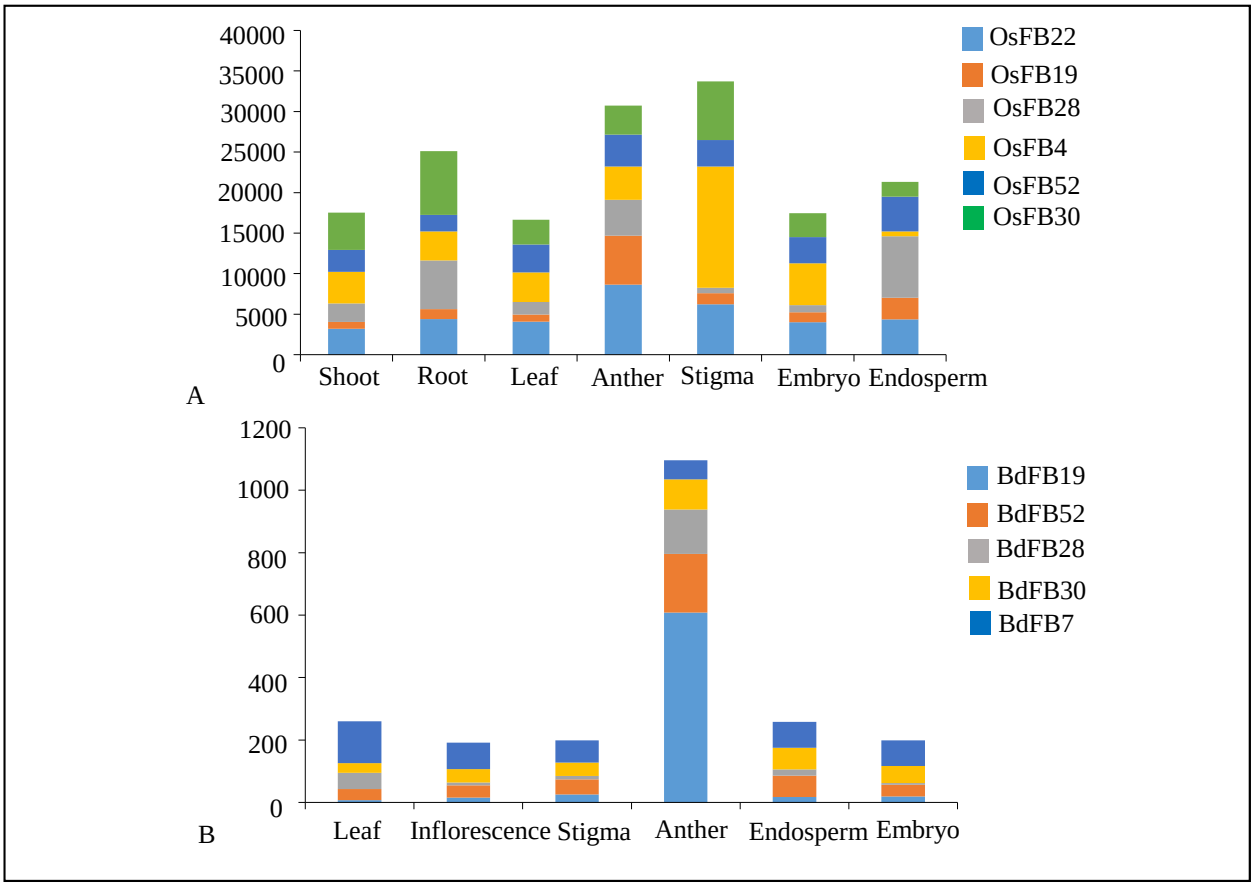

Figure 4.9. *In silico* gene expression analysis of *FBox* genes from (A) rice and (B) *Brachypodium*. Selection of F Box genes was performed based on their RPKM assessed expression values across different vegetative and reproductive tissues. The X axis denotes tissue stages and the Y axis indicates their RPKM values.

4.8.3. *In silico* analyses of promoter elements of candidate *Fbox* genes

In addition to *in silico* expression analyses, the 1500 bp upstream sequences of the candidate rice and *Brachypodium Fbox* genes were analyzed to identify pollen/anther specific cis elements

(Figure X). Primarily, three pollen specific cis elements, POLLEN1LELAT52 (AGAAA), GTGANTG10 (GTGA) and QELEMENTZMZM13 (AAGTCA) were present in rice *Fbox* promoters. Except for *OsFBL52*, all these elements were distributed throughout the 1500 bp upstream region (**Figure 4.10**). Most of these cis elements were also present in Brachypodium *F box* genes. However, the QELEMENTZMZM13 element was absent in majority *BdFBL* genes except *BdFBL7*.

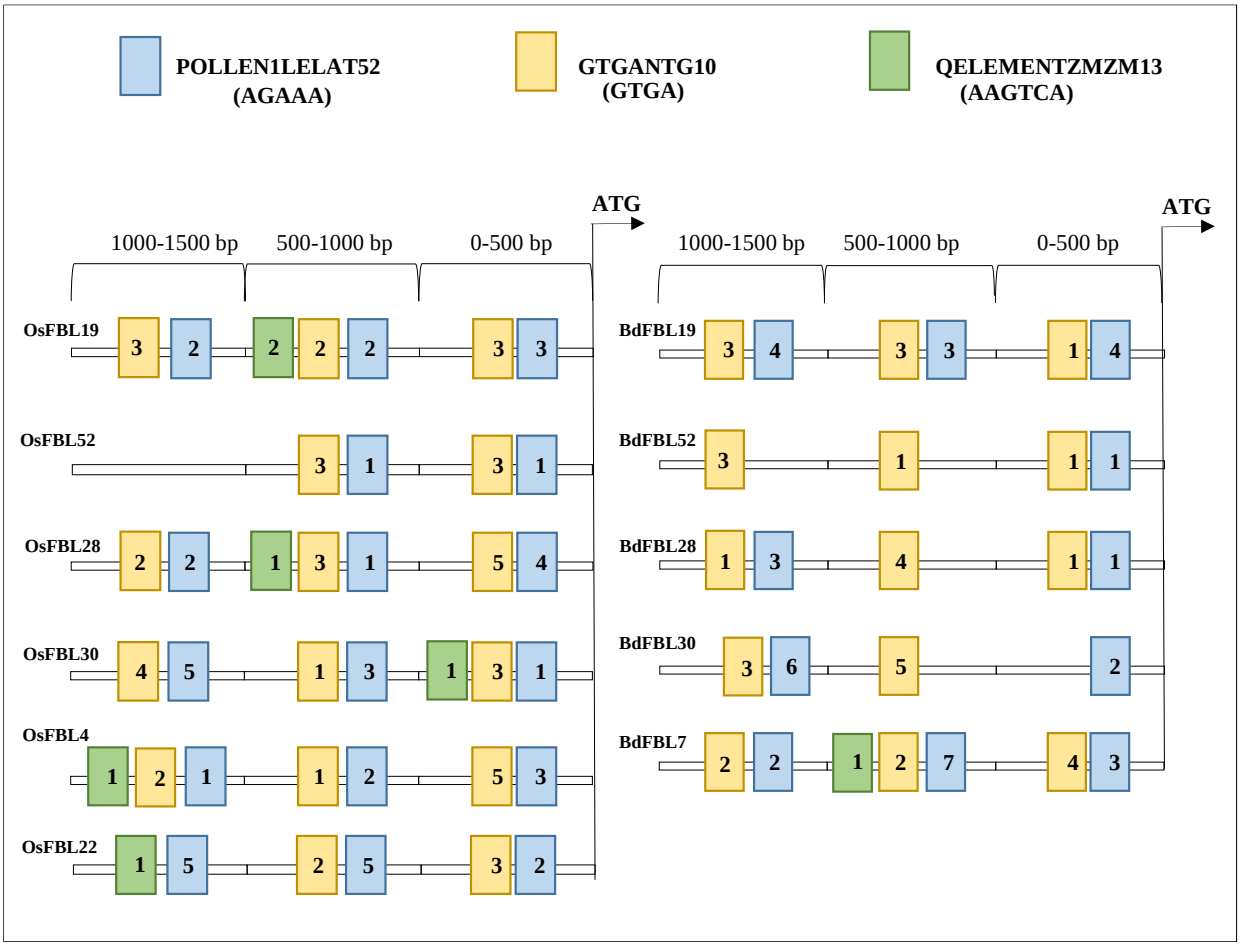

Figure 4.10. *In silico* identification of anther/pollen specific cis elements in the promoters of selected rice and *Brachypodium FBL* genes.

4.8.4. Isolation and molecular characterization of *RNS1* homolog from *B. tulda*

A single copy of *RNS1* homolog have been cloned and sequenced from *B. tulda*. Sequence based homology search analysis using the partial *BtRNS1* sequence in NCBI database revealed highest

homology with the homolog of *Triticum dicoccoides* (XP_037484051.1, 79.89%). Predicted models of *RNS1* genes obtained from different Poaceae plants suggested that the homolog contain two exons and a single intron. *In silico* comparison of the amino acid sequences revealed that the molecular weight of these RNS1 proteins varied from 18464.81 to 29303.57 Da and the lengths were 162 to 266. Interestingly, both the SRNase and RNase like amino acid patterns are present in all the bamboo *RNS1* homologs (**Figure 4.11A**). In pattern I, the two important residues- histidine and glycine (FGI**HG**LWPNY), responsible for RNase function and compatibility reaction, respectively were conserved across all the selected monocot species (Figure. 4.12A). However, few changes in the amino acid sequences were observed in case of pattern II, III and IV. (**Figure 4.11A**).

4.8.5. Study on the phylogenetic relationship of *S-RNase* gene in comparison to the homologous sequences from related monocot species

In order to study the phylogenetic relationship of *B. tulda RNS1* gene to related monocotyledonous species, NJ tree was constructed based on the deduced protein sequences (**Figure 4.11B**). Two main clusters were observed. The cluster 1 was comprised of members of the Poaceae family and cluster 2 consisted of non-grass species such as *Z. marina*, *S. polyrhiza*, *A. comosus*, *P. dactylifera*, *C. simplicifolius* and *A. officinalis* (**Figure 4.11B**). All the bamboo *RNS1* homologs formed a bamboo specific cluster.

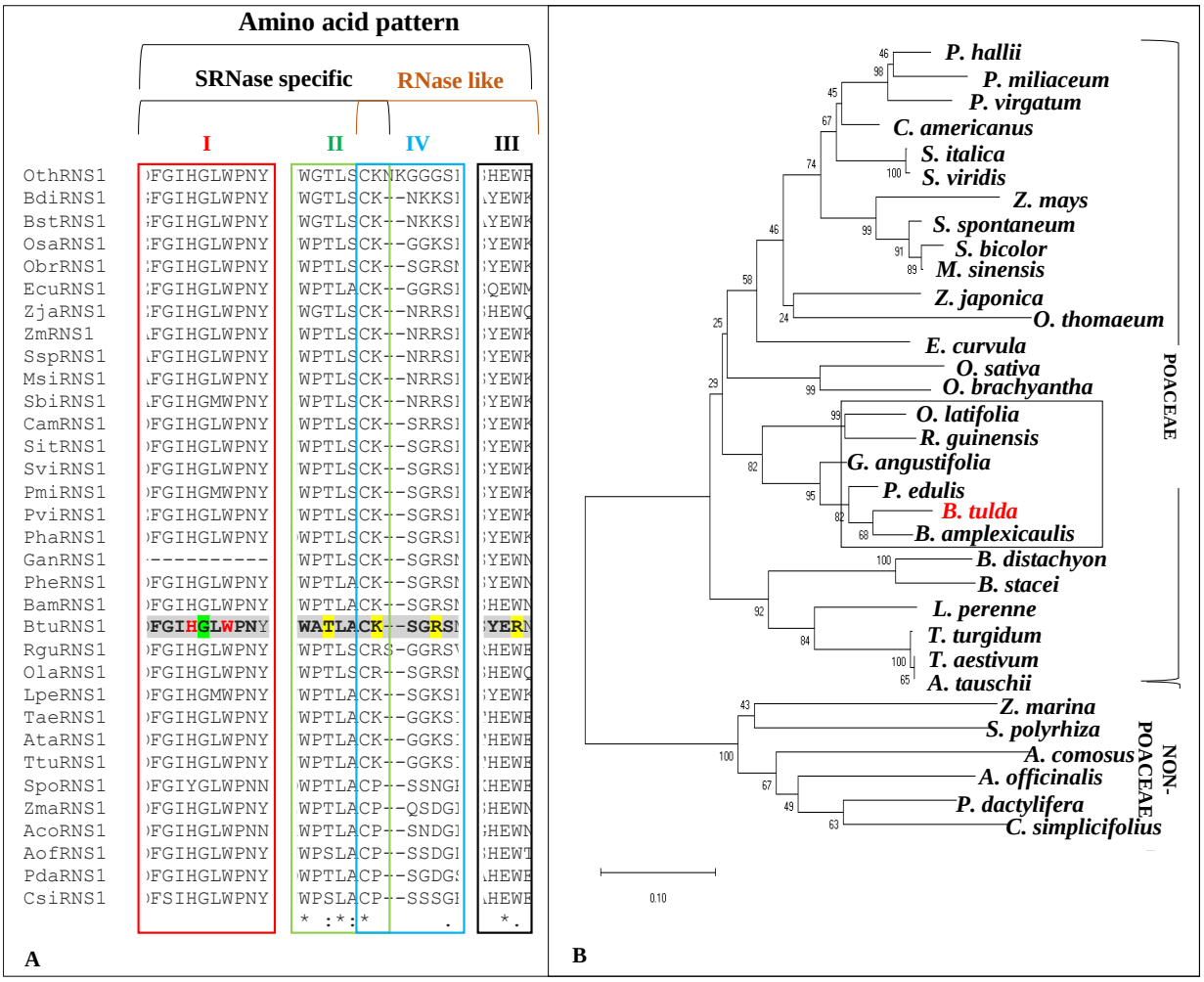

Figure 4.11. Identification of determining amino acid sequence patterns and phylogenetic analysis of *BtRNS1* gene in comparison to other monocotyledonous plants. (A) Multiple alignment of BtRNS1 proteins with homologous monocotyledonous sequences and comparison of amino acid patterns (SRNase specific- I, II and RNase like- III, IV). The BtRNS1 sequence is highlighted in grey. Any deviation in the amino acid residues within a pattern are highlighted in yellow; important residues linked to RNase specific function is highlighted in red and residues for sexual compatibility is highlighted in green. (B) Constructed Neighbour Joining (NJ) tree of the BtRNS1 homolog Bootstrap value used was 3000.

4.8.6. Tissue specific expression analyses of *RNS* genes by real time RTq-PCR analyses

The transcriptional abundance of the selected *RNS* genes were measured in seven different flowering and non flowering tissue tissues. Among the selected four genes, *RNS1* gene was primarily expressed in the male and female reproductive organs compared to the other genes (**Figure 4.12**).

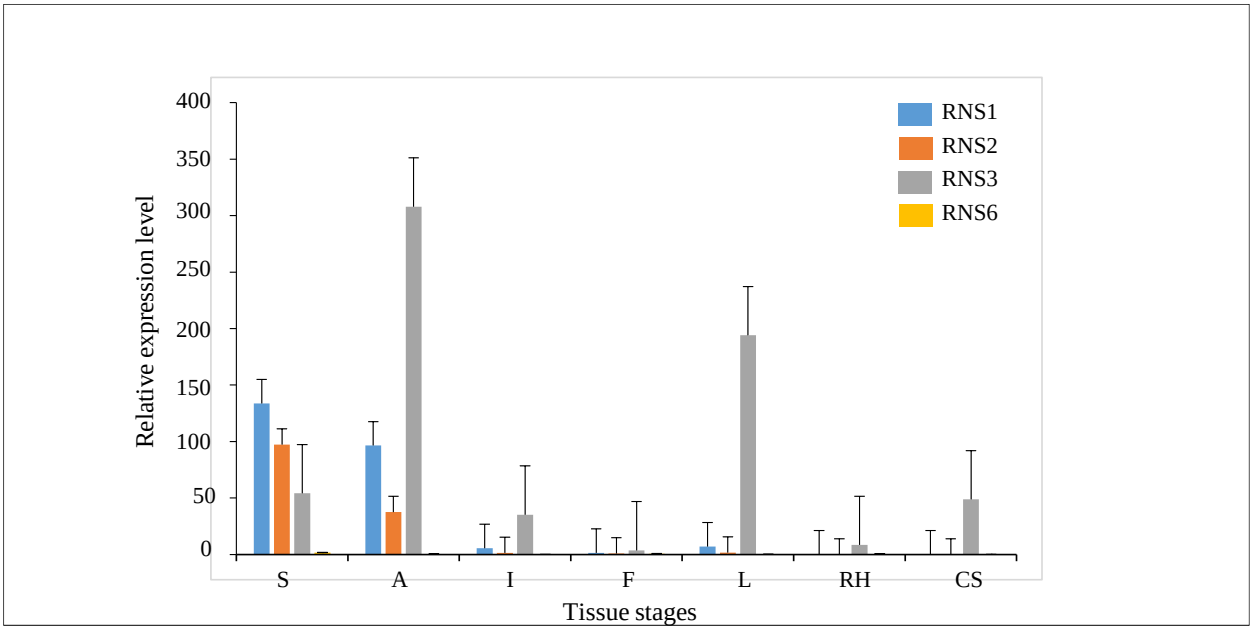

Figure 4.12. Gene expression analysis of candidate RNaseT2 family genes in *B. tulda*. The *UBQ5* gene was used as reference gene and root as the calibrator. The $2^{-\Delta\Delta CT}$ method was used to measure the relative fold of the targeted genes. The fold change value of the three biological replicates ±SE were represented in each bar. Abbreviations used: S: stigma, A: anther, I: internode, F: floral bud, L: leaf, RH: rhizome, CS: culm sheath

Interestingly, the transcript abundance of this gene was also higher in stigma than anther tissue. This suggests its possible involvement in stigma specific function and therefore it was selected as the candidate gene for PCR isolation and sequence characterization from *B. tulda*.

Objective IV. *In silico* identification and sequence characterization of floral organ specific MADS box family genes in bamboo and other grasses

4.9. PCR isolation, sequencing and molecular characterization of *BtMADS14* gene

4.9.1. *In silico* identification of flowering associated MADS box family genes from all the sequenced genomes of Poaceae plants

At least one homologous gene for each of the MADS box family members from the six flower specific clades (*AGAMOUS, AG; AGAMOUS LIKE 2, AGL2; AGL6; DEFICIENS/ GLOBOSA, DEF/GLO; GMM13/B$_{sister}$; SQUAMOSA, SQUA*) could be identified from majority species. However, except for the AP1/SQUA clade, number of genes present in an individual clade is lower in bamboo than rice. For example, in case of AG clade, only a single homolog was identified in *P. edulis* (**Table 4.3**). In AGL2 clade, only two homologs were identified in *P. edulis, O. latifolia* and *R. guianensis,* four in *B. amplexicaulis* and one in *G. angustifolia* (**Table 4.4**). Similarly, for AGL6 clade, a single homolog was identified in *P. edulis, O. latifolia* and *G. angustifolia* (**Table 4.5**). In case of DEF/GLO clade, a single gene was identified in *G. angustifolia*, while it was two in *P. edulis, O. latifolia* and *R. guianensis* (**Table 4.5**). For GMM13 clade, only a single homolog was identified in case of *P. edulis*, whereas two for the other four bamboos (**Table 4.6**). Finally, in case of SQUA clade, only two homologous genes were identified in *P. edulis, R. guianensis* and *G. angustifolia* (**Table 4.7**).

Table 4.3. *In silico* identification of AG genes across five bamboo genomes. BLASTP analysis was performed where O. sativa sequences were used as query. Criteria for BLASTP analyses and subsequent selection of homologs were: identity (≥40%), E values (e-10) and query coverage (≥40%). For any individual gene if more than one BLAST hits were obtained, the top BLAST hit was selected for further analyses.

Bamboo species	MADS3 homolog	MADS58 homolog	MADS13 homolog	MADS21 homolog
Phyllostachys edulis	PH01002127G0260	PH01002127G0260	PH01002127G0260	PH01002127G0260
Olyra latifolia	Ola036689.1	Ola026000.1	Ola030284.1	Ola023380.1
Bonia amplexicaulis	Bam023611.1	Bam037944.1	Bam005823.1	Bam021423.1
Guadua angustifolia	Gan023296.1	Gan001620.1	Gan000617.1	Gan000617.1

Raddia guianensis	Rgu014860.1	Rgu014860.1	Rgu002247.1	Rgu002511.1

Table 4.4. *In silico* identification of AGL2 genes identified across five bamboo genomes. BLASTP analysis was performed where *O. sativa* sequences were used as query. Criteria for BLASTP analyses and subsequent selection of homologs were: identity (≥40%), E values (e-10) and query coverage (≥40%). For any individual gene if more than one BLAST hits were obtained, the top BLAST hit was selected for further analyses.

Bamboo species	MADS1 homolog	MADS5 homolog	MADS34 homolog	MADS7 homolog	MADS8 homolog
Phyllostachys edulis	PH01001952G0190	PH01001952G0190	PH01000306G0610	PH01000306G0610	PH01000306G0610
Olyra latifolia	Ola001944.1	Ola001944.1	Ola001944.1	Ola000561.1	Ola000561.1
Bonia amplexicaulis	Bam030728.1	Bam001790.2	Bam023088.1	Bam021236.3	Bam021236.3
Guadua angustifolia	Gan000617.1	Gan000617.1	Gan000617.1	Gan000617.1	Gan000617.1
Raddia guianensis	Rgu003296.1	Rgu003296.1	Rgu003296.1	Rgu007745.1	Rgu007745.1

Table 4.5. *In silico* identification of AGL6 and DEF/GLO genes identified across five bamboo genomes. BLASTP analysis was performed where *O. sativa* sequences were used as query. Criteria for BLASTP analyses and subsequent selection of homologs were: identity (≥40%), E values (e-10) and query coverage (≥40%). For any individual gene if more than one BLAST hits were obtained, the top BLAST hit was selected for further analyses.

Bamboo species	AGL6		DEF/GLO		
	MADS6 homolog	MADS167 homolog	MADS16 homolog	MADS2 homolog	MADS4 homolog
Phyllostachys edulis	PH01000306G0610	PH01000306G0610	PH01001878G0200	PH01000317G0080	PH01001878G0200
Olyra latifolia	Ola020254.1	Ola020254.1	Ola004315.1	Ola016612.1	Ola016612.1

Bonia amplexicaulis	Bam010961.1	Bam022193.1	Bam013132.1	Bam030437.1	Bam026194.1
Guadua angustifolia	Gan017073.1	Gan017073.1	NH	Gan016557.1	Gan016557.1
Raddia guianensis	Rgu007626.1	Rgu003296.1	Rgu008982.1	Rgu021882.1	Rgu021882.1

Table 4.6. *In silico* identification of GMM13 genes identified across five bamboo genomes . BLASTP analysis was performed where *O. sativa* sequences were used as query. Criteria for BLASTP analyses and subsequent selection of homologs were: identity (≥40%), E values (e-10) and query coverage (≥40%). For any individual gene if more than one BLAST hits were obtained, the top BLAST hit was selected for further analyses.

Bamboo species	MADS29 homolog	MADS30 homolog	MADS31 homolog
Phyllostachys edulis	PH01000338G0120	PH01000338G0120	PH01000338G0120
Olyra latifolia	Ola005387.1	Ola005387.1	Ola022392.1
Bonia amplexicaulis	Bam029747.1	Bam012981.1	Bam029747.1
Guadua angustifolia	Gan029910.1	Gan029910.1	Gan007464.1
Raddia guianensis	Rgu010127.1	Rgu010127.1	Rgu018984.1

Table 4.7. *In silico* identification of SQUA genes identified across five bamboo genomes. BLASTP analysis was performed where *O. sativa* sequences were used as query. Criteria for BLASTP analyses and subsequent selection of homologs were: identity (≥40%), E values (e-10) and query coverage (≥40%). For any individual gene if more than one BLAST hits were obtained, the top BLAST hit was selected for further analyses.

Bamboo species	MADS14 homolog	MADS15 homolog	MADS18 homolog	MADS20 homolog

Phyllostachys edulis	PH01000606G0250	PH01000606G0250	PH01000306G0610	PH01000006G0210
Olyra latifolia	Ola026653.1	Ola015294.1	Ola019483.1	Ola009746.1
Bonia amplexicaulis	Bam023087.1	Bam037248.1	Bam024416.1	Bam017915.2
Guadua angustifolia	Gan000617.1	Gan003796.1	Gan003796.1	Gan003796.1
Raddia guianensis	Rgu011099.1	Rgu011099.1	Rgu011099.1	Rgu012908.1

4.9.2. Study on the phylogenetic relationship of flowering associated MADS box family genes among Poaceae grasses

In order to understand the phylogenetic relationship of the flowering associated MADS box genes among the closely related Poaceae members, NJ trees were constructed based on the protein sequences. In case of each of the six MADS box gene clades containing multiple genes, homologous genes from related grass species formed gene specific clusters. In case of AG, AGL6, GMM13 and SQUA clades, the homologous bamboo genes formed bamboo specific cluster. Interestingly, for AGL2 clade genes the *P. edulis*, homologs were quite distant from that of its related orthologs in other grasses and was not grouped with any sub clusters**.** Formation of four different clusters were observed among members of SQUA clade, where four gene specific groups- *MADS14, 15, 18* and *20* were formed (**Figure. 4.13**). Cluster I was composed of *MADS20* gene homologs from all related grasses along with five bamboos. Similarly, cluster II, III and IV were comprised of MADS18, 15 and 14 genes respectively. Further, a bamboo specific sub cluster was formed within cluster I (**Figure. 4.13**). However, no such clear sub clustering were observed in the other gene clusters.

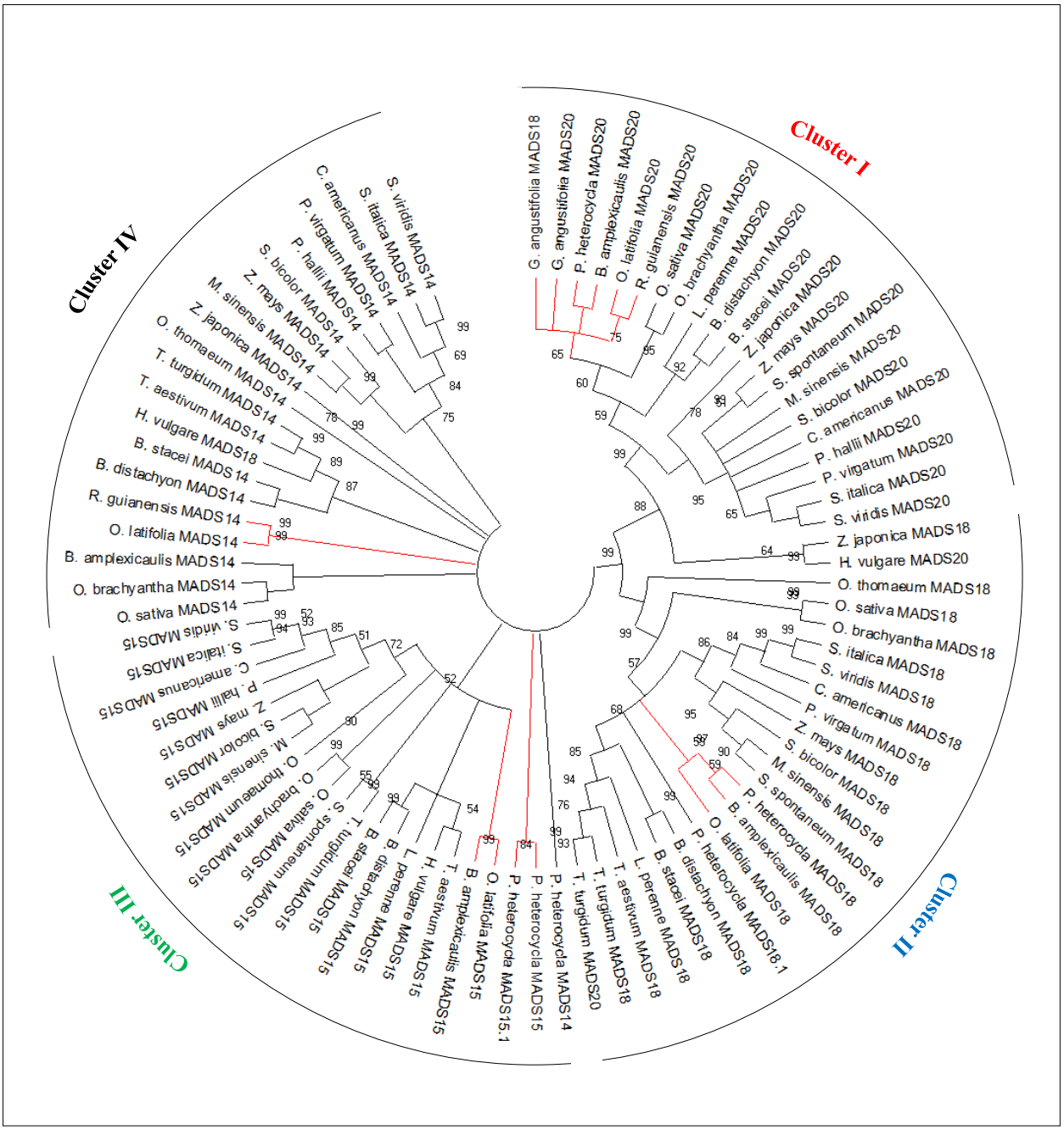

Figure 4.13. Phylogenetic analysis of members of SQUA clade genes among different grass species. Phylogenetic analysis of members of SQUA clade genes. Neighbour Joining (NJ) tree was constructed after multiple aligning full-length protein sequences in Mega 10.0 and subsequently constructing the phylogenetic tree using default parameters and bootstrap value 3000.

4.9.3. Identification and molecular characterization of *B. tulda MADS14* gene

The *AP1/FUL* family member, *MADS14* have been isolated from *B. tulda* by designing degenerate primers as mentioned in section 3.11. The obtained sequences were deposited to NCBI database (BtMADS14- KX186751). Sequence homology search in the NCBI non-redundant database revealed that the highest homology of *BtMADS14* (97%) was with the temperate bamboo *P. edulis* (AHM92070.1). The *BtMADS14* coding sequence is 735 nucleotide long and the predicted protein is 244 amino acid long. The predicted protein contains a highly conserved MADS domain, K domain, I domain and variable C terminal ends with the FUL motif (L/MPPWML/V). The MADS domain is composed of 58 amino acids, entire K domain has 66 amino acids and in between these two regions I domain of 33 amino acids is located. The molecular weight of BtMADS14 proteins is 28242.39 kDa.

4.10. Study on the phylogenetic relationship of *BtMADS14* gene in comparison to the homologous sequences from related monocot species

In order to study the phylogenetic relatedness of *BtMADS14* gene to other monocotyledonous species, NJ trees were constructed based on multiple aligned protein sequences (**Figure 4.14**). Two major clusters comprising of members of the Poaceae and non-Poaceae families were obtained (**Figure 4.14**). The Poaceae specific cluster was again subdivided into two sub clusters-sub cluster 1 was composed of species from BOP clade containing Pooideae, Oryzoideae and Bambusoideae grasses (*B. tulda*, *B. oldhamii*, *P. heterocycle*, *P. praecox*, *D. latiflorus* and *F. nitida*; **Figure 4.14**). On the other hand, subcluster 2 was only comprised of grasses from the PACMAD clade. The *BtMADS14* gene was most closely related to *P. edulis MADS14* gene (**Figure 4.14**).

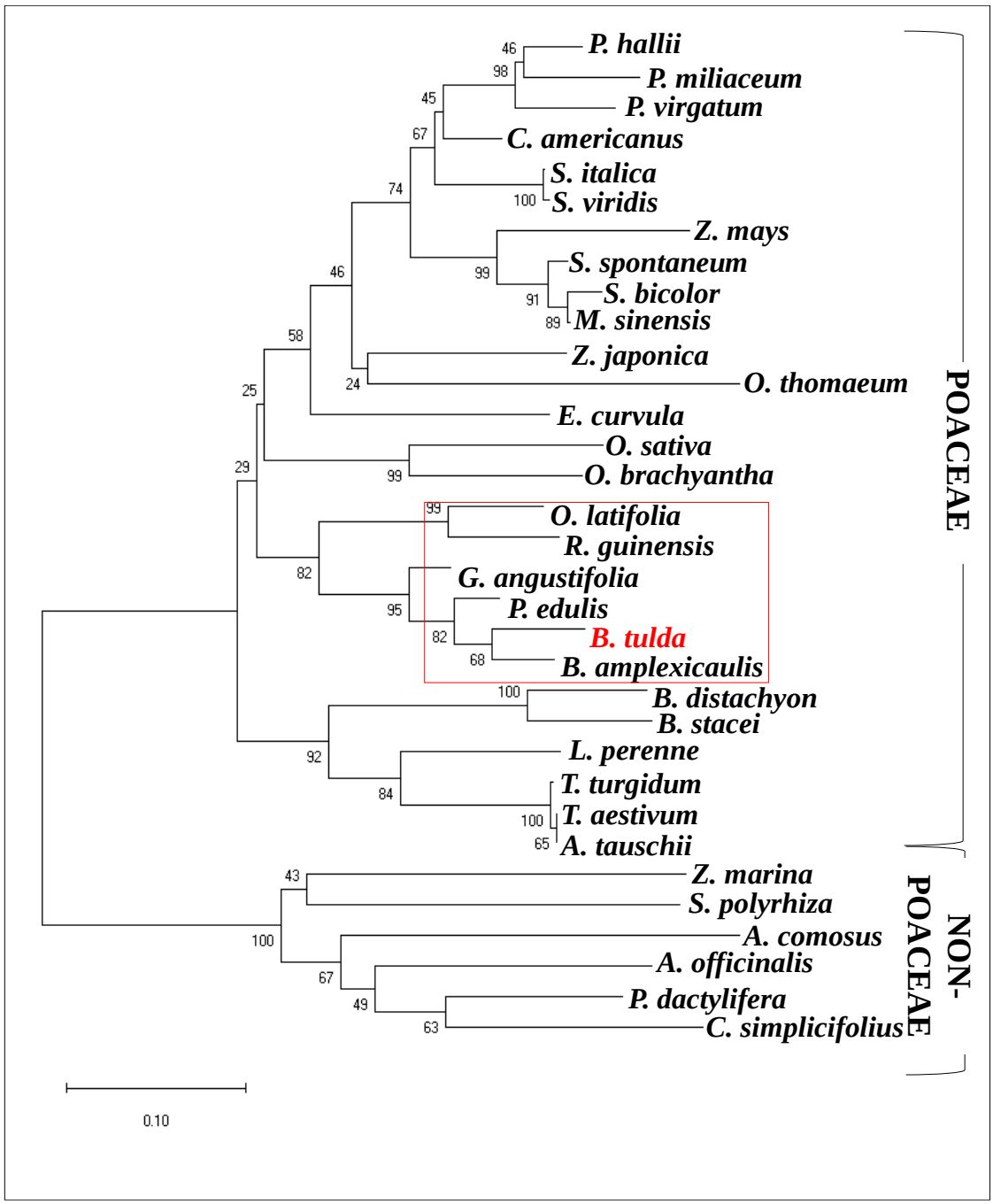

Figure 4.14. Phylogenetic analysis of *BtMADS14* gene with different monocotyledonous plants. Neighbour Joining (NJ) tree of the *BtMADS14* homologs was constructed after multiple aligning full-length protein sequences in Mega 10.0 and subsequently constructing the phylogenetic tree using default parameters and bootstrap value 2000.

4.11. Tissue specific expression analyses of *BtMADS14, 15 and 18* genes by real time RTq-PCR analyses

The *BtMADS14* and *BtMADS15* genes were primarily expressed in floral buds and internode (**Figure 4.15**). The *BtMADS14* gene was also highly expressed in the early staged inflorescence bud (E) and the level gradually decreased in the mature buds (**Figure 4.15)**. On the other hand, both *BtMADS15* and *BtMADS18* showed relatively higher expression in leaves obtained from flowering culm (YLF) than that of non-flowering one (YLN, **Figure 4.15)**.. However, the transcript abundance of *BtMADS18* gene was mostly below the threshold level for majority tissues studied.

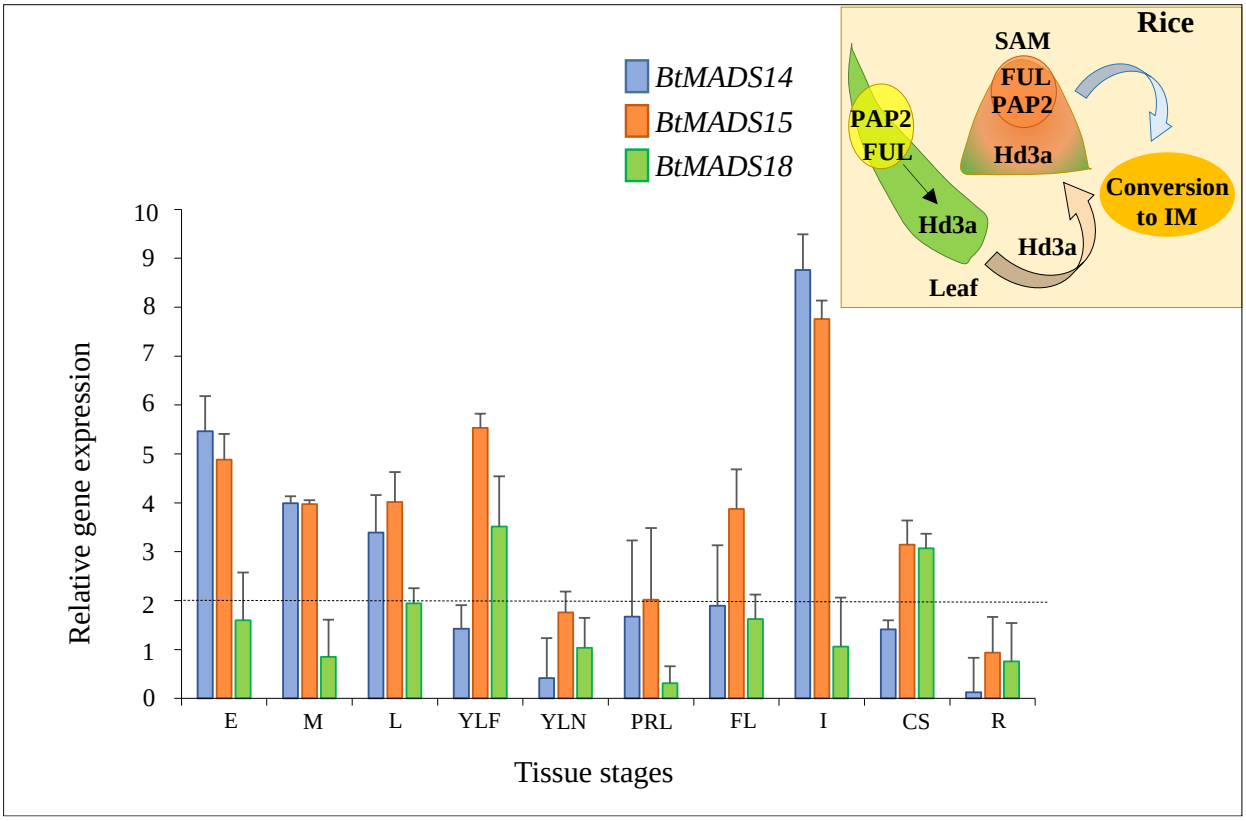

Figure 4.15. Study of expression levels of *AP1/FUL* genes in different flowering and non-flowering tissue stages. Transcript expression of *eIF4α* was used to normalize expression data of the targeted MADS box genes. The relative fold change was calculated by $2^{-\Delta\Delta CT}$ method using the expression level observed in rhizome as the calibrator. Each bar represents mean of three biological replicates ±SE. Pictorial representation of role of *AP1/FUL* gene in both leaves

and SAM during floral transition of rice in the inset. The set cut off for gene expression fold is marked as a dotted line. Abbreviations used: CS- culm sheath, I: internode, YLF- young leaf from flowering culm, YLN- young leaf from non-flowering culm, PFL: possible flag leaf, FL: flag leaf, E: early staged inflorescence bud, M: middle staged inflorescence bud, L: late staged inflorescence bud, R: root, SAM: shoot apical meristem, Hd3a: *HEADING DATE 3A*, PAP2: *PANICLE PHYTOMER 2, FUL: FRUITLULL.*

4.12. *In silico* analyses of promoter sequences in comparison to homologs from other closely related monocot species

To understand the possible cause of expression divergence among members of class A genes, 1500 bp upstream regions of the three *AP1/FUL* genes were obtained from the sequenced bamboo *P. edulis* and their cis element composition were compared with that of other related species. *In silico* search of cis elements revealed that the promoter regions of all three *AP1/FUL* genes are primarily composed of four types of cis elements, which are regulated by light, development, hormone and stress. In addition, core promoter elements such as TATA box and CAAT box were also detected (**Table 4.8**).

Table 4.8. *In silico* identification of cis elements in the promoters of *MADS14, MADS15* and *MADS18* genes.

Species / Gene	Cis elements	*O. sativa*	*T. aestivum*	*B. distachyon*	*B. stacei*	*P. edulis*	*Z. mays*	*P. halli i*	*S. italica*	*S. viridis*	*S. bicolor*
MADS14	Total	19	28	27	15	22	25	27	31	31	31
	Light	7	7	9	7	5	14	14	14	12	2
	Development	1	6	3	2	2	0	3	0	1	11
	Stress	2	5	6	2	9	9	8	13	14	11
	Hormone	9	6	9	4	6	2	2	4	4	7
MADS15	Total	23	22	18	14	18	11	27	21	27	29

	Light	10	12	7	4	5	2	14	9	14	10
	Development	2	4	1	4	1	3	3	0	1	3
	Stress	4	4	4	5	2	5	8	11	11	12
	Hormone	7	2	6	1	10	1	2	1	1	4
MADS18	Total	20	29	29	25	3	24	24	25	20	18
	Light	11	13	13	11	2	5	10	13	7	8
	Development	0	0	1	1	0	0	5	1	1	1
	Stress	4	9	10	8	1	2	6	9	8	5
	Hormone	5	7	5	5	0	17	3	2	4	4

The light responsive element is primarily abundant in all three genes across different species, and both the *MADS14* and *MADS15* genes of *P. edulis* contain similar number of these elements in their promoters. Interestingly, the number of cis elements related to hormone was higher in PeMADS15 than other related species. Among all the selected species, the total number of cis elements in the promoter of MADS18 gene was highest in both *T. aestivum* and *B. distachyon*; whereas it was lowest with only three cis elements on *P. edulis* (**Table 4.8**). In addition to this, presence of development related cis elements were absent in the *PeMADS18* promoter, which was unlike both *PeMADS14* and *PeMADS15* genes (**Table 4.8**).

4.13. *In silico* analyses of structural motifs identified in homologs of closely related monocot species

In order to understand the sequence diversity of the *BtMADS14* protein compared to related monocotyledonous species, *in silico* prediction of conserved motifs was performed. A total of 15 conserved motifs were identified. The motif 1 containing MADS domain was present across all the monocotyledonous species except for *A. officinalis*. Other than this, few conserved motifs were also present in the K and I domains. Most of the unconserved motifs were located in the variable C domain.

5. Discussion

5.1. Identification of suitable reference gene is mandatory to perform gene expression studies in tropical bamboos

The primary advantage of real time RT-qPCR method is its high accuracy and specificity and thus extensive studies have been performed to identify suitable reference genes that are stable across diverse experimental conditions (species: Morgante et al. 2011; developmental stage: Fan et al. 2013, Li et al. 2016; tissue: Reddy et al. 2016; different seasons: Kim et al. 2015; and diverse environmental conditions: Silva et al. 2014, De Andrade et al. 2017). However, despite these unusual features, studies related to the underlying molecular mechanism is still scarce in tropical bamboos. Thus, this study focuses on the identification of suitable reference genes in widely grown tropical woody bamboos across four species, nine organs and three developmental stages, which will be helpful for proper data normalization during gene expression analyses. It was observed that a universal single reference gene could not normalize data in all the tissue stages and species. This observation revealed the importance of standardization of reference gene prior to any gene expression analysis.

5.2. The two different inflorescence types differ in their time of occurrence and rate of seed setting

In grasses, spikelet inflorescence containing florets is the primary structural unit with grass specific outer organs and conserved male and female organs (Yoshida and Nagato, 2011). Unlike grasses, bamboos display diversity in their inflorescence morphology where two different spikelets, called true spikelet and pseudospikelet are observed (Wysocki et al., 2016, Wang and Li, 2019). This study revealed that true spikelet was more abundant during early stages of flowering cycle, whereas pseudospikelet was more abundant in the later stage (Chakraborty et al., 2021, accepted for publication). Other than this, the rate of overall seed setting was also significantly higher in pseudospikelet than spikelet. Since the rate of pollen fertility and seed-setting is low in bamboo, the high number of seed production in pseudospikelet inflorescence suggests that it to be a reproductive strategy to ensure increased seed formation prior to the monsoon season.

5.3. The *BtRNS1* gene possibly plays an important role during sexual incompatibility in *B. tulda*

Since flowering time is unusually extended in bamboo, possibility of genetic variability due to sexual reproduction is less frequent. Thus, bamboos ensure genetic variability by maintaining a high rate of cross-pollination. In this study, the *in vivo* pollination experiment performed on two distant populations of *B. tulda* suggested this is primarily a cross pollinated species. Wide variations exist across bamboo species with respect to the predominance of self- vs. cross pollination (Ramanayake and Weerawardene, 2003; Kitamura and Kawahara, 2011; Liu et al., 2014; Mizuki et al., 2014; Xie et al., 2019). However, studies on the molecular regulation behind such complex genetic compatibility in grasses is broadly unknown. Grasses display a GSI based incompatibility system (Conner, 1979). One of the most characterized GSI is mediated by SRNase based mechanism, which has been characterized well in diverse dicotyledonous plants (Igic et al., 2007; Li et al., 2015; Claessen et al., 2019). This study identifies RNase T2 family genes across 40 different Poaceae and non Poaceae species including five bamboo species. Moreover, one *RNS1* homolog was also identified from *B. tulda*. Phylogenetic analysis revealed a clear bamboo specific cluster formation and the *BtRNS1* gene was more closely related to *B. amplexicaulis* than other bamboos. Detailed tissue specific gene expression analysis revealed that *BtRNS1* was primarily expressed in the reproductive tissues, particulalrly in stigma. Thus, *BtRNS1* may be associated with self incompatibility response in bamboos.

5.4. MADS box genes associated with flowering are present in bamboo

The genetic regulation of floral organ differentiation in grasses is controlled by the ABCDE classes of MADS box genes and has been studied in a wide range of species (Callens et al., 2018). A few studies have also been performed in different bamboo species (Wang et al. 2014; Shih et al. 2014; Zhu et al. 2016; Cheng et al. 2017; Jiao et al. 2019). This study identifies genes belonging to 21 MADS box families across six different flower associated clades in 23 grass species including five bamboo species. The number of homologs varied in different bamboos and in some cases, the bamboo sequences were more closely related to members of the Pooideae subfamily than rice. In addition to this, a single copy of *AP1/FUL* family gene *MADS14* was isolated from *B. tulda*, which possessed high sequence similarity with other monocotyledonous

genes and contains biologically active domain. Phylogenetic analysis suggested *BtMADS14* was more closely related to rice, compared to other species. Further expression analyses of *BtMADS14* gene along with two other *AP1/FUL* genes in *B. tulda* in diverse tissues revealed their important role in flowering.

6. Significance and future directions

This study aims to fill the gap in understanding bamboo flower development at morphological, developmental and genetic level. In objective I, identification of suitable reference genes to normalize gene expression data in bamboos was performed. The stability analyses of ten candidate reference genes were performed at diverse organ, developmental stages and species level. The stable reference gens identified in this study will be useful in future gene expression analyses performed on tropical bamboos.

In objective II, the two different inflorescences of bamboos were carefully studied throughout an entire flowering cycle to compare their reproductive performance and significance. Visible differences were observed between the two inflorescence types with respect to their frequency at different times of a flowering cycle and number of seeds produced by them. Further, in order to understand the development of inflorescence buds, SEM analyses were performed. Future studies may aim to understand ecological significance of these two types of inflorescences.

The main purpose of objective III was to study the biology and genetics of pollen compatibility in *B. tulda*. *In vivo* self and cross pollination experiments performed among two geographically distant populations of *B. tulda* suggested that the plant is primarily cross pollinated species. This study also characterized RNase T2 and *Fbox* gene families to illuminate their possible involvement in controlling genetic compatibility in bamboos. Future studies may conduct more extensive gene expression analyses of *BtRNS1* gene along with *in planta* transformation to pinpoint its specific role in genetic compatibility.

In objective IV, developmentally regulated MADS box genes obtained from five sequenced bamboo genomes were characterized by various *in silico* tools. The *AP1/FUL* member genes *BtMADS14* and *BtMADS15* were identified from *B. tulda.* Their expression analysis in different tissues suggested possible involvement of *BtMADS14* and *15* in early stages of inflorescence

development. In contrast, overall very low transcript abundance was observed for *BtMADS18* gene. Future studies may focus to characterize promoters of these genes in *B. tulda*, which may identify the molecular factors regulating their expression divergence. Also, ectopic expression of these genes in rice may suggest their precise role in flower or vegetative development.

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7. Appendix I

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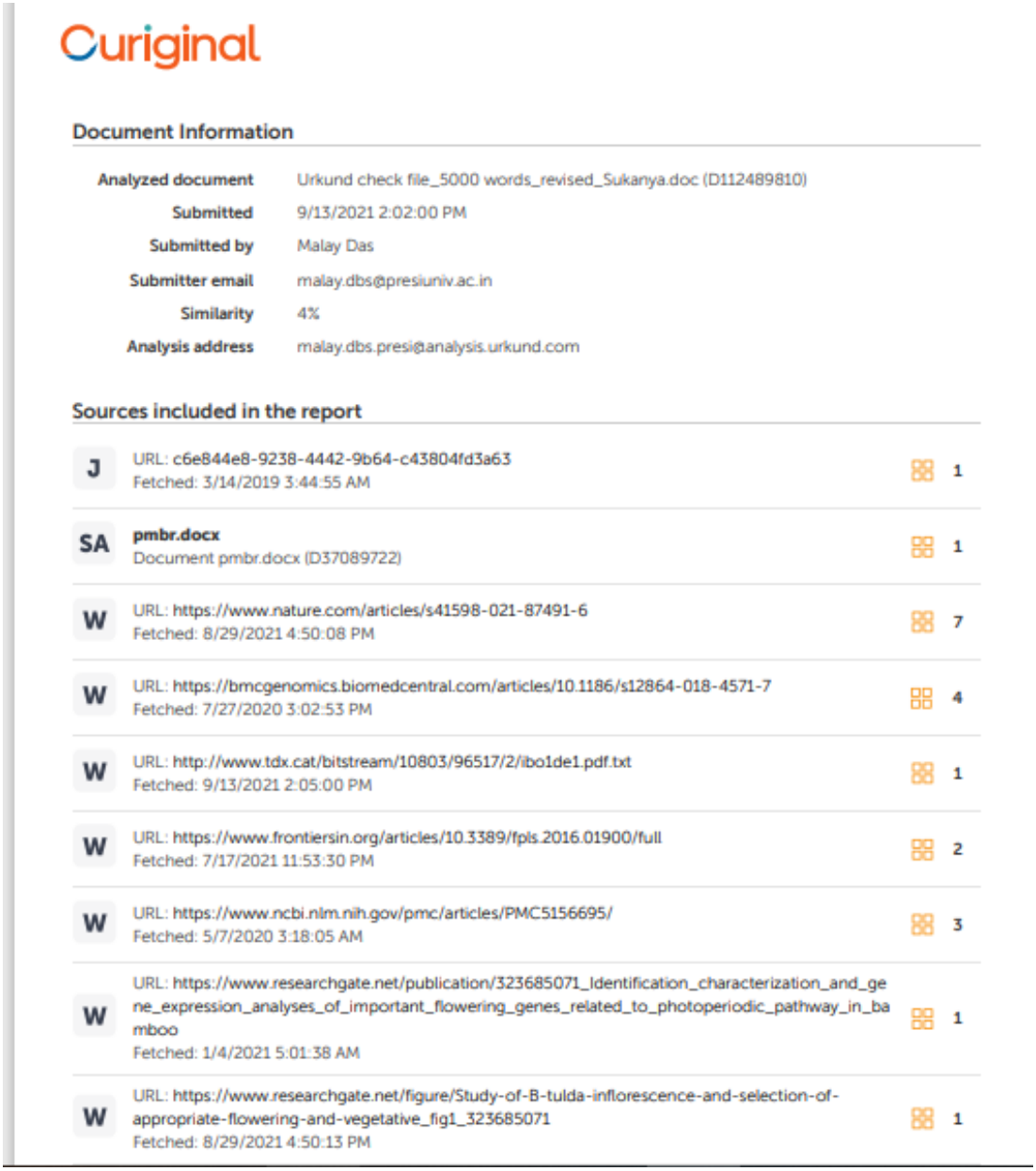

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7.2. Certificates of conferences attended and first pages of published manuscripts

Plant Physiology Forum

24th S.M. Sircar Memorial Conference

This is to certify that

Mr. / Ms/Dr/Prof. Sukanya Chakraborty

from Dept. of Life Science, Presidency University *has actively participated in the* 24th *S.M. Sircar Memorial Conference organised by Plant Physiology Forum held on* 23.02.18 *2018 in the Base Institute Auditorium at Main Campus Kolkata - 700 009.*

Prof. Swapan Kumar Datta
President, PPF

Prof. Prabir Kumar Saha
Treasurer, PPF

Prof. Dibyendu Narayan Sengupta
Secretary, PPF

International Conference
on
Algae, Fungi and Plants: Systematics to Applications
(AFPSA-2020)
January 24 –25, 2020
Organized by
CAS, Department of Botany, University of Calcutta
In collaboration with
Botanical Survey of India
100TH ANNIVERSARY OF CALCUTTA UNIVERSITY HERBARIUM (CUH)

Prof./Dr./Mr./Ms. Sukanya Chakraborty of Presidency University has presented a paper entitled Understanding the biology of Sexual compatibility in bamboo in the International Conference on "*Algae, Fungi and Plants: Systematics to Applications (AFPSA-2020)*" held on January 24-25, 2020 at CAS-VII, Department of Botany, University of Calcutta, Kolkata, India.

Dr. Debabrata Maity
Convener

Prof. K. Acharya
Convener & HoD

NATIONAL CONFERENCE
ON
SCIENCE & TECHNOLOGY: RURAL DEVELOPMENT
20TH & 21ST JANUARY, 2020
ORGANISED BY
ISCA, KOLKATA CHAPTER & SURENDRANATH COLLEGE, KOLKATA

CERTIFICATE OF PARTICIPATION & POSTER PRESENTATION

This is to certify that Prof./Dr./Mr./Ms Tumpa Chakraborty
of Presidency University
participated & presented a poster in the two-days National Conference
organised by ISCA, Kolkata Chapter & Surendranath College, Kolkata.

PROF. TUSHAR KANTI GHOSH
Convener, ISCA, Kolkata Chapter

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Bamboo Flowering from the Perspective of Comparative Genomics and Transcriptomics

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Bamboos are an important member of the subfamily Bambusoideae, family Poaceae. The plant group exhibits wide variation with respect to the timing (1–120 years) and nature (sporadic vs. gregarious) of flowering among species. Usually flowering in woody bamboos is synchronous across culms growing over a large area, known as gregarious flowering. In many monocarpic bamboos this is followed by mass death and seed setting. While in sporadic flowering an isolated wild clump may flower, set little or no seed and remain alive. Such wide variation in flowering time and extent means that the plant group serves as repositories for genes and expression patterns that are unique to bamboo. Due to the dearth of available genomic and transcriptomic resources, limited studies have been undertaken to identify the potential molecular players in bamboo flowering. The public release of the first bamboo genome sequence *Phyllostachys heterocycla*, availability of related genomes *Brachypodium distachyon* and *Oryza sativa* provide us the opportunity to study this long-standing biological problem in a comparative and functional genomics framework. We identified bamboo genes homologous to those of *Oryza* and *Brachypodium* that are involved in established pathways such as vernalization, photoperiod, autonomous, and hormonal regulation of flowering. Additionally, we investigated triggers like stress (drought), physiological maturity and micro RNAs that may play crucial roles in flowering. We also analyzed available transcriptome datasets of different bamboo species to identify genes and their involvement in bamboo flowering. Finally, we summarize potential research hurdles that need to be addressed in future research.

Keywords: bamboo, flowering pathways, genes, drought, plant age, future research

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INTRODUCTION

Flowering is one of the most important adaptations in the evolution of land plants. Numerous studies have been performed on annual, herbaceous model plants from dicotyledonous (*Arabidopsis*, *Antirrhinum*) and monocotyledonous (*Oryza*) groups to identify and characterize important floral pathway genes (Putterill et al., 2004; Colasanti and Coneva, 2009). However, the majority of commercially important plants are perennial and there remains a gap in translating knowledge gained from annual, model plants to perennial plants. Therefore, increasing research attention is being paid to perennial plants. While poplar (Jansson and Douglas, 2007) and white spurge have emerged as model perennial dicotyledonous plants (Anderson et al., 2007), research on perennialism remains elusive in monocots.

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Identification of candidate reference genes in tropical bamboos stable across species, tissues, and developmental stages

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Abstract

Bamboo possesses many unique physiological characteristics, but the molecular understanding of many of these processes remains poorly understood till to date. One major reason is unavailability of sufficient sequence and expression data. Selection of suitable reference genes is pivotal to initiate any gene expression analyses. Although, suitable reference genes have been identified in the temperate bamboo *Phyllostachys edulis*, it has not been done for tropical bamboo. In this study, expression stability of 10 candidate reference genes were investigated in 4 widely grown tropical bamboo species (*Bambusa tulda*, *B. balcooa*, *B. bambos*, and *B. vulgaris*), different organs (young leaves from flowering and non-flowering culms, flag leaf (leaf just below the mature inflorescence), possible flag leaf (leaf covering the immature inflorescence), culm sheath, internode, root, rhizome, and inflorescence bud), different parts (basal, middle, and tip regions of leaf; internodes located in the basal, middle, and tip region of the branch, and developmental stages early, middle, and late inflorescence buds) by using 3 reliable computational tools (*geNorm*, *NormFinder*, and *RefFinder*). A universal single reference gene for normalization of gene expression data was not identified. However, the *eukaryotic initiation factor 4α* (*eIF4α*), *clathirin adaptor complexes medium subunit* (*CAC*), and *nucleotide tract-binding protein* (*NTB*) were found stable in the selected organs across different bamboo species. On the other hand, *eIF4α* ranked top when different organs and *peptidyl prolyl cis-trans isomerase/cyclophilin* (*CYP), eukaryotic elongation factor 1α* (*eEF1α*) and *ubiquitin 5 (UBQ5)* ranked top when different developmental stages of *B. tulda* were analyzed. Taken together, this study not only identifies reference gene/s that are stable across species, organs, and developmental stages of bamboo, but it also assesses the impacts of major contributing factors regulating expression stability of the reference genes.

Additional key words: *Bambusa balcooa*, *B. bambos*, *B. tulda*, *B. vulgaris*, *CAC*, *CYP*, *eEF1α, eIF4α*, *NTB*, *UBQ5*.

Introduction

Bamboos are members of the subfamily *Bambusoideae*, family *Poaceae*. They are profusely growing in different regions of Asia, America, and Africa (Clark *et al*. 2015). A well-developed bamboo culm offers a multiple usage, such as in construction purposes, charcoal production, and a raw material for paper and pulp production, whereas young shoots are used as vegetable (Lobovikov *et al.* 2007, Owen 2015). Research on bamboo has traditionally been restricted to phylogenetic studies (Zhang 2000, Clark *et al.* 2007, Das *et al.* 2008), standardization of *in vitro* regeneration methods (Das and Pal 2005, Ramanayake *et al.* 2006, Yuan *et al.* 2017), ecological studies on the unusually long flowering cycle (Janzen 1976, Marchesini *et al.* 2009, Sertse *et al.* 2011),

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Abbreviations: 18S - 18S ribosomal RNA; ACT7 - actin 7; CAC - clathirin adaptor complexes medium subunit; CS - culm sheath; CYP - peptidyl prolyl *cis-trans* isomerase/cyclophilin; E - early staged inflorescence bud; eEF1α - eukaryotic elongation factor 1α; eIF4α - eukaryotic initiation factor 4α; FL - flag leaf; GAPDH - glyceraldehyde-3-phosphate dehydrogenase; I - internode; L - late staged inflorescence bud; M - middle staged inflorescence bud; NTB - nucleotide tract-binding protein; PFL - possible flag leaf; R - root; RH - rhizome; RPKM - reads per kilobase million;TOC1 - timing of CAB expression1; UBQ - ubiquitin; YLF - young leaf from flowering culm; YLN - young leaf from non-flowering culm.

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